

EFFECTS OF FREEZING ON THE MECHANICAL PROPERTIES OF ENGINEERED TISSUES

by

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## ABSTRACT

### EFFECTS OF FREEZING ON THE MECHANICAL PROPERTIES OF ENGINEERED TISSUES

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Cryopreservation is used to preserve cells and tissues at low temperatures for short and long term uses. Some examples of where cryopreservation is used include the storage of organs, blood, embryos, and artificial tissues. A current challenge of cryopreservation is being able to understand and quantify the effects of freezing/thawing on the functional properties of tissues such as mechanical properties. This study investigated the changes in viscoelastic parameters of engineered tissues before and after freezing by performing creep-relaxation tests using a dynamic mechanical analyzer. Three types of engineered tissues were studied with varying cell and collagen concentrations for two different cell types, human dermal fibroblast (BR5) and breast cancer cells (MCF-7). The three types of engineered tissues include 3mg/ml of collagen with 200,000 cells/ml, 3mg/ml of collagen with 400,000 cells/ml, and 6mg/ml of collagen with 200,000 cells/ml. A Kelvin-Voigt model was used to characterize the viscoelastic properties of the engineered tissue. The viscoelastic properties of all engineered tissues were evaluated and compared to characterize the effects of freeze/thaw, cell concentration, collagen concentration and cell types. The results were discussed considering the structural changes of the extracellular matrix due to each parameter.

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## CHAPTER 1

### INTRODUCTION

#### 1.1 Background of Cryopreservation

Cryobiology consists of two things, cryosurgery and cryopreservation. Cryosurgery uses freezing to damage cells or tissue and cryopreservation uses freezing to preserve cells or tissues and has the potential for long-term storage [1]. Cryopreservation consists of freezing cells or tissues to low temperatures to slow down all chemical, physical, and biological processes [2]. Some examples of where cryopreservation is used is the cryopreservation of human embryos for in vitro fertilization [3], to store blood cells, sperm, and eggs [4], engineered tissues such as skin grafts and artificial cartilages [2], and for storage and transport of artificial tissues [5]. Some preservation techniques are currently developed for tissue preservation including cryopreservation, hypothermic preservation, vitrification, freeze-drying, and slow-freezing [6]. Vitrification is where water is vitrified into a glass state without the formation of ice crystals [2]. Among these techniques, cryopreservation is a leading candidate for tissue preservation. However, cryopreservation of tissues is more complicated than that of cells in suspension since cells within the tissue can experience different physical and chemical changes during cryopreservation. These changes include the spatiotemporal changes of cryoprotective agents (CPAs) and temperature. Since the primary goal of cryopreservation of tissue is for the maximum recovery, functionality, and viability of cells within the tissue [2], understanding the effects of those spatiotemporal changes on the functionality and viability is critical.

Thus, one of the most significant challenges of cryopreservation of tissues is to preserve the functional properties of tissues such as mechanical properties. This challenge of maintaining the functionality of tissues during cryopreservation is associated with controlling the biophysical events during the loading and unloading of cryoprotective agents (CPAs), during

freezing, and finally controlling the temperature response during freeze/thaw [7]. Among various functional properties, mechanical properties have been mostly studied to characterize the effects of cryopreservation protocols as reviewed in Han and Bischof (2004). Some examples of these mechanical properties studied are physical dimensions, ultimate tensile strength, and maximum stiffness. In spite of these previous studies, there is no clear understanding of the relationship between biophysical events and the mechanical properties of tissue [7]. Besides preserving native tissues, needs to cryopreserve various engineered tissues are emerging as tissue engineering technology advances. Tissue engineering is needed for medical implants to solve problems such as organ deficiencies. The major obstacle in this is storage of ET and preservation [2].

### 1.2 Literature Review: Current Status

The effect of cryopreservation on the mechanical properties of tissues is currently being studied. A previous study on the effects of cryopreservation on mechanical properties froze pig femoral arteries in a controlled rate freezer using a probe and passively thawed them to room temperature. The arteries were also subjected to uniaxial tensile testing. Some changes seen in the mechanical properties after freezing consist of the loss of smooth muscles cell viability, damage to the extracellular matrix (ECM), changes to alignment caused by ice growth, the redistribution of water, the reduction in weight, and finally an increase in the artery diameter [8].

Han and Bischof (2004) reviewed several tests and changes in mechanical properties of preserved tissues. The first example is a smooth muscle contractility test of a rabbit carotid artery. The changes in mechanical properties seen were endothelial cell damage and low recovery of smooth muscle contractility. Another example is the diameter measurement under a biaxial loading of canine sphenous veins. The change was a gradual loss of biaxial compliance [7].

Teo (2009) developed a new experimental method used to measure spatiotemporal deformation during freezing of an ET. This method obtained quantitative data on cell-fluid-matrix interaction during freeze/thaw. The ET was composed of human foreskin fibroblasts and type 1 rat tail collagen. This experiment showed that ET expanded and compressed during freezing. The expansion occurs just after the phase change interface and the unfrozen region compresses before the phase change interface. Some changes seen after freeze/thaw in this study were that the frozen/thawed ET is thinner than the unfrozen ET and that the weight of the ET decreases after it has been frozen and thawed [6].

Dehoyos (2009) studied the effects of freeze/thaw on the ECM were studied. The physical condition of collagen fibrils of the ECM were studied before and after freeze/thaw for three types of ETs with different collagen and cell concentrations of BR5 cells and type I rat tail collagen. Some changes seen after the freeze/thaw procedure in this study were that the fibril area decreases, the mean void area increases, the ECM is less compact, and the fibril diameter increases. This means that less collagen fibrils are present after freeze/thaw. Mean void area is the void size between fibrils. This study concluded that the freezing process changes individual fibrils and increases their size [9].

### 1.3 Objective of the Present Study

The objective of the present study is to quantify changes of the viscoelastic properties of ET before and after freeze/thaw. This will ultimately help cryopreservation practice in maintaining functionality and viability of ETs. Since the mechanical properties are thought to be affected by microscopic biophysical phenomena during freeze/thaw, ETs with different tissue microstructure are studied. These tissue structural parameters are the cell concentration, collagen concentration, and the cell type. The effects of these parameters on the viscoelastic parameters are also quantified and tested on both unfrozen and frozen/thawed ETs.

## CHAPTER 2

### THEORETICAL BACKGROUND

#### 2.1 Problem Statement

Engineered tissues (ET) are composed of an extracellular matrix (ECM) with collagen fibrils, cells, and interstitial fluid, and considered as viscoelastic materials. The problem statement is to quantify the viscoelastic properties of ETs before and after freeze/thaw for cryopreservation of functional ETs. A simplified system is selected to model the viscous and elastic behavior of the ET. This model will be discussed later in this section.

#### 2.2 Literature Review: Viscoelastic Material Models

Models used to demonstrate the viscoelastic response of ETs are composed of a combination of springs and dashpots. The combination of springs and dashpots is used to form the viscous and elastic components of the stress-strain curve. An example of one model is composed of four elements including two springs and two dashpots. This model includes a dashpot that is in series with two parallel elements, another dashpot and a spring, and again in series with another spring [10]. This model was used to represent linear behavior and therefore was not chosen because the behavior of the ET is nonlinear. Another study used a three parameter model composed of two springs and one dashpot. This included a spring in parallel to a spring and a dashpot [11]. This study modeled the viscoelastic behavior of chondrocyte cells and was considered a standard linear solid model and was also not chosen for the model of the ET. Chondrocytes are from osteoarthritic cartilage. Finally, another study used the Kelvin-Voigt Model to model a nonlinear behavior using a four element model. This model included two springs and two dashpots. This model was chosen to model the ET and is further discussed in section 2.3 [12].

### 2.3 Kelvin-Voigt Model

Figure 2.1 shows the mechanical schematic of the spring-dashpot model that was used for the Kelvin-Voigt Model. The spring elements represent the elastic components and the dashpot elements represent the viscous components. This is also known as a dashpot in series with a Voigt body.

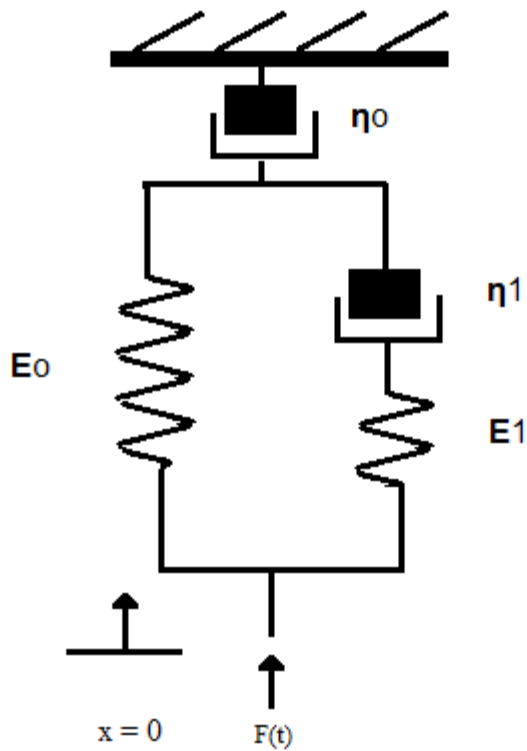


Figure 2.1 Kelvin-Voigt Model.

Equations 2.1 and 2.2 [12] are used to represent the strain-stress behavior according to the Kelvin-Voigt model:

$$\frac{\epsilon(t)}{\sigma_0} = \frac{1}{E_0} \left[ 1 - \frac{E_1}{E_0 + E_1} \exp\left(\frac{-t}{\tau}\right) \right] + \frac{t}{\eta_0}$$

Eq. (2.1)

$$\tau = \frac{\eta_1(E_0 + E_1)}{E_0 E_1} \quad \text{Eq. (2.2)}$$

Where  $\varepsilon(t)$  is strain,  $\sigma$  is stress, and  $\tau$  is the relaxation time. As discussed earlier the  $E_0$  and  $E_1$  represent the elastic components and the  $\eta_0$  and  $\eta_1$  represent the viscous components.  $E_0$  is the equilibrium modulus,  $E_0 + E_1$  is the instantaneous modulus,  $\eta_0$  is the effective viscous friction coefficient. Figure 2.2 shows a creep-relaxation curve for the Kelvin-Voigt model. Regime I is the elastic response, Regime II is the relaxation regime, and Regime III is the viscous flow regime. The relaxation time is the time required to transition from elastic regime to the viscous regime [12]. The viscoelastic response is cell and tissue type dependent. This study used BR5 and MCF-7 cells with collagen type I.

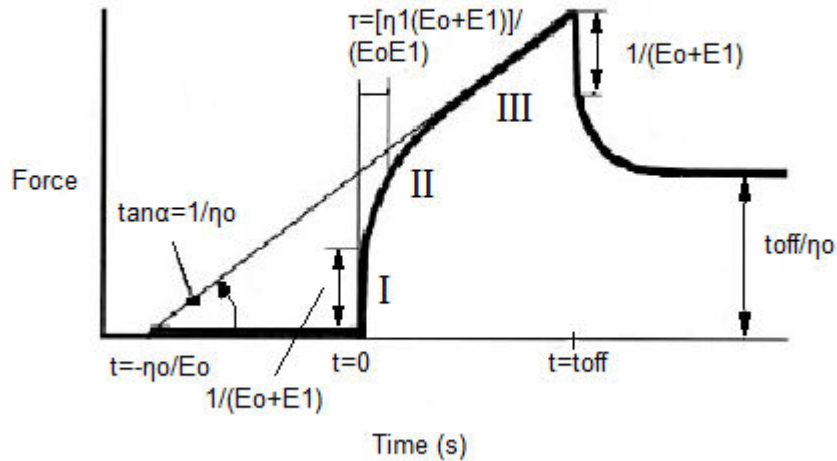


Figure 2.2 Creep-Relaxation Curve of Kelvin-Voigt Model redrawn [12]

CHAPTER 3  
MATERIALS AND METHODS

3.1 Cell Culture

Human breast adenocarcinoma MCF-7 cells and human dermal fibroblast BR5 cells were used. The MCF-7 cells were cultured in a corning flask with culture medium for five to seven days depending on when the cells filled the flask. The culture medium is composed of 500 ml of Advanced DMEM/F12 basal medium, 25 ml of fetal bovine serum (FBS), 5 ml of L-glutamine, and 5 ml of Penicillin/Streptomycin (P/S). Otherwise mentioned, all chemicals were purchased from Invitrogen (Carlsbad, California). To collect the cells, the flask was then rinsed with 8 ml of Dulbecco's Phosphate Buffered Saline (DPBS) twice. The flask was then rinsed with 0.5 ml of Trypsin. Next 2 ml of Trypsin was added to the flask and put in the incubator at 37°C for 10 minutes or until the cells have detached from the wall of the flask. Next 8 ml of culture medium was added to the flask. The cell suspension was then transferred into a 15 ml centrifuge tube and put in the centrifuge at 1000 rpm for 3 minutes. The old culture medium was removed without disturbing the cell pellet and 5 ml of new culture medium was added and the suspension was mixed. Next the amount of cells/ml was determined by counting the cells using a haemocytometer (Bright-Line counting chamber, Fisher Scientific) and an inverted microscope. The appropriate amount of cell suspension was then removed with a pipette and centrifuged again at the same specifications. The culture medium was removed and the remaining cell pellet was used in the gel.

The same procedure was used for the BR5 cells with the exception of the culture medium composition and the centrifuge settings. The culture medium is composed of 500 ml of Advanced DMEM/F12 basal medium, 50 ml of fetal bovine serum (FBS), 5 ml of L-glutamine,



and 5 ml of Penicillin/Streptomycin (P/S). The centrifuge settings are set at 2000 rpm for 4 minutes.

### 3.2 Engineered Tissue Preparation

The engineered tissue (ET) was constructed by seeding either of collected cells in collagen solution which is composed of 10X MEM, sterile 1.0N NaOH, 0.1 M HEPES, P/S, L-Glutamine, FBS, sterile dH<sub>2</sub>O, and rat tail collagen (Rat Tail Collagen Type I, BD Biosciences, Bedford, MA). The ET is prepared in a 15 ml centrifuge tube in an ice water bath. MEM, NaOH, HEPES, P/S, L-Glutamine, FBS, and dH<sub>2</sub>O are added to the tube and mixed. Next the solution is added to the cell pellet and mixed again. Next the rat tail collagen is added to the solution and mixed carefully to avoid bubbles. The new solution is then put in a chamber slide (Lab-Tek II, Nunc, Naperville, IL), covered, and placed in the incubator for one hour. The ET is then removed from the incubator and the ET is detached from the walls of the chamber slide. Finally 2 ml of cultured medium is added to the chamber slide and put back in the incubator for 24 hours.

The collagen and cell concentrations were varied for each cell type. The ET studied were 1) 3mg/ml of collagen with 200,000 cells/ml, 2) 3mg/ml of collagen with 400,000 cells/ml, and 3) 6mg/ml of collagen with 200,000 cells/ml. For this study, 3mg/ml of collagen with 200,000 cells/ml is called the Control, 3mg/ml of collagen with 400,000 cells/ml is called high cell concentration (HCELL), and 6mg/ml of collagen with 200,000 cells/ml is called high collagen concentration (HCOLL).

### 3.3 Freezing Procedure

The excess medium and sidewall of the chamber slide is then removed and the ET is then placed on a directional solidification stage [6]. The solidification stage is composed of two blocks that are temperature controlled. These blocks are cooled by using gaseous liquid

nitrogen and have a 6 mm gap between them. The heat controller number one was set to 4°C and the heat controller number two was set to -20°C. An amount of 200 µl of 100% Ethanol is applied to the tops of the blocks to prevent ice formation on the blocks and to create contact between the chamber slide and the blocks. The ET was then frozen directionally from -20°C to 4°C. . The chamber slide with the ET is then removed from the freezing stage and allowed to thaw for 10 minutes to room temperature.

### 3.4 Creep-Relaxation Test Using DMA

A creep-relaxation test is then performed on the unfrozen and frozen/thawed ET using Dynamic Mechanical Analysis (DMA Q800, TA instruments).

#### *3.4.1 Set Up*

The ET is cut to a diameter of 19.5 mm and placed into the bottom cup of the compression clamp of the DMA as seen in Figure 3.1. The plate and cup can be seen in Figure 3.2. The top plate of the clamp is then lowered until it barely touches the top surface of the ET and then locked into place. After the furnace of the DMA is closed, the test is set up by using TA Instrument Explorer where the thickness of the ET is measured to be approximately 1 mm.

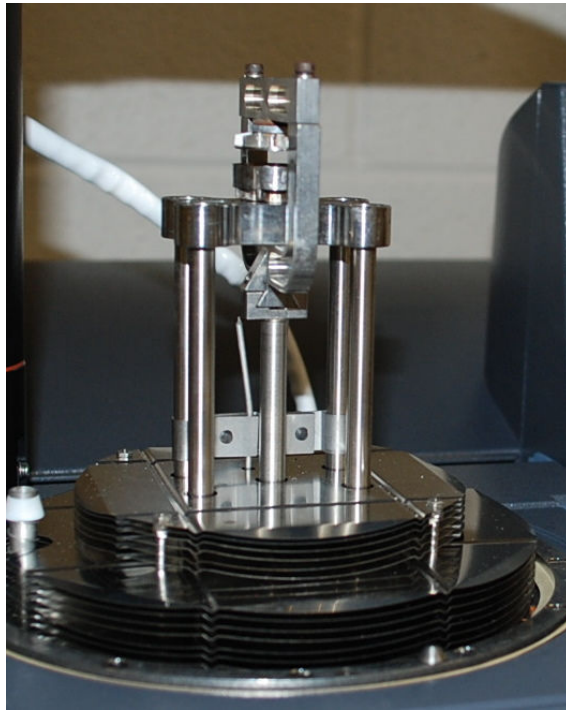


Figure 3.1 Compression Clamp.

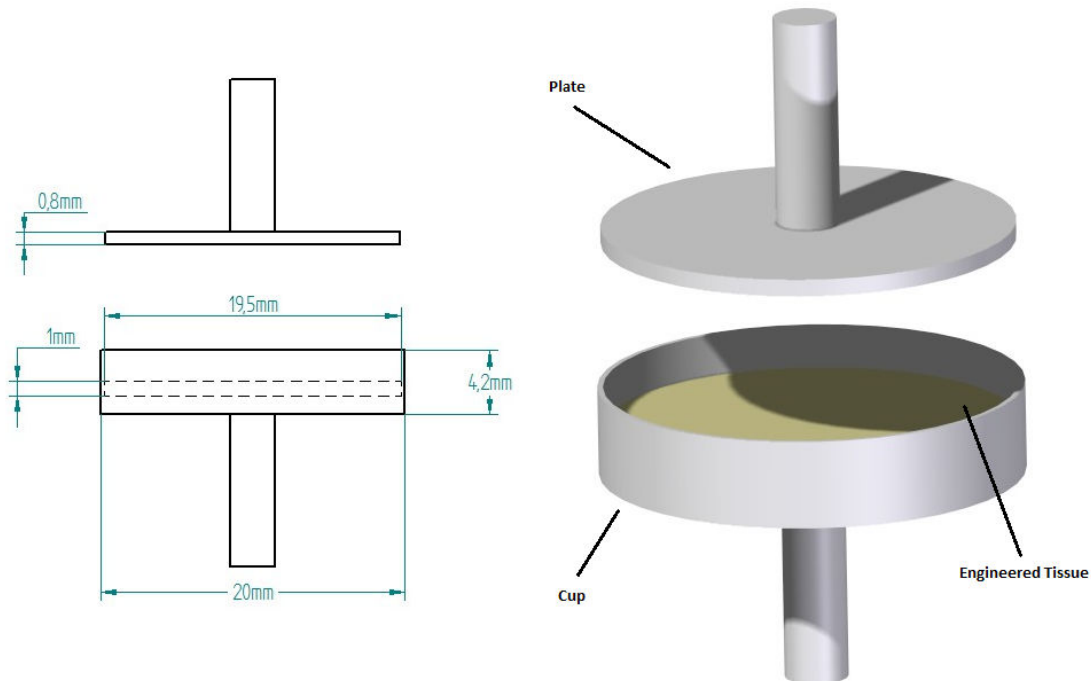


Figure 3.2 Plate and Cup.

### 3.4.2 Testing

TA Instrument Explorer is used to set up the test of the DMA. The first testing parameter is to set the test mode to DMA Controlled Force for a round specimen. A custom test is set up with a preload force of 0N which remains isothermal for one minute. Next a constant force of .01N is applied for five minutes and finally the force is removed and is isothermal for one minute. The creep-relaxation tests were performed at room temperature.

The DMA measured time, stress, strain, and displacement. First a baseline measurement for each tissue type was taken. The baseline measurement was a DMA test of the ET without an applied force to measure the effect of just the clamp weight. Next a creep-relaxation test was performed as described earlier. The baseline measurement was subtracted from the creep-relaxation test. Next the compliance, strain divided by stress, was calculated.

The new beginning time was set to zero where the force was first applied and the data was analyzed for 50 seconds. Microsoft excel equation solver was used to solve for the viscoelastic parameters in equations 2.1 and 2.2 along with nonlinear regression analysis [14].

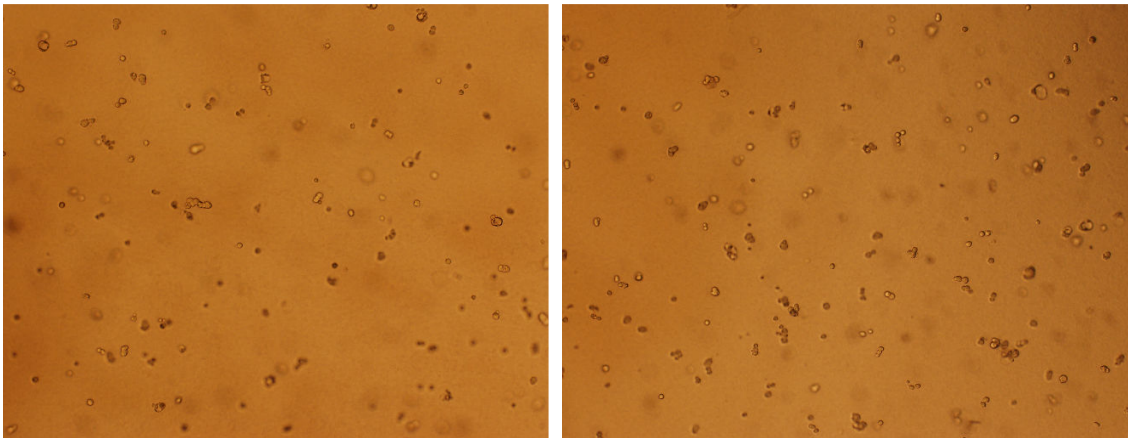
CHAPTER 4  
RESULTS AND DISCUSSION

4.1 Microscopic Changes

Images of three different types of ET samples were taken with an Olympus DP70 camera on an Olympus IX71 microscope. These images were taken before and after freezing and before and after the DMA creep-relaxation test. The different cell and collagen concentrations were studied for both cell types including 1) Control, 2) HCELL, and 3) HCOLL as discussed in section 3.2.

*4.1.1 Micrographs of ET Before and After DMA*

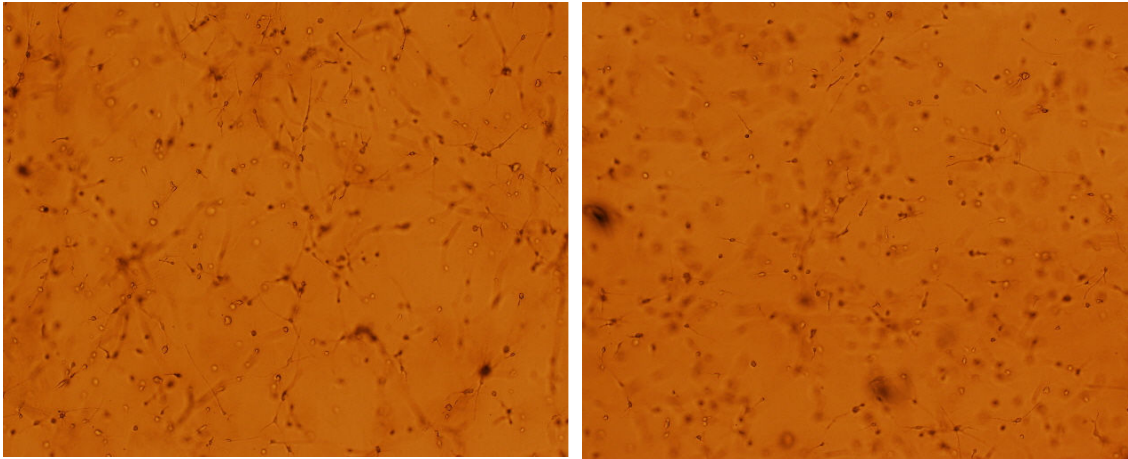
Figure 4.1 and Figure 4.2 show MCF-7 and BR5 respectively before and after the DMA test was performed at a collagen concentration of 3mg/ml and a cell concentration of 200k/ml. No damage can be seen after the test was performed.



(a)

(b)

Figure 4.1 3mg/ml 200k/ml MCF-7 Microscopic Images at 4X Magnification (a) before DMA (b) after DMA.



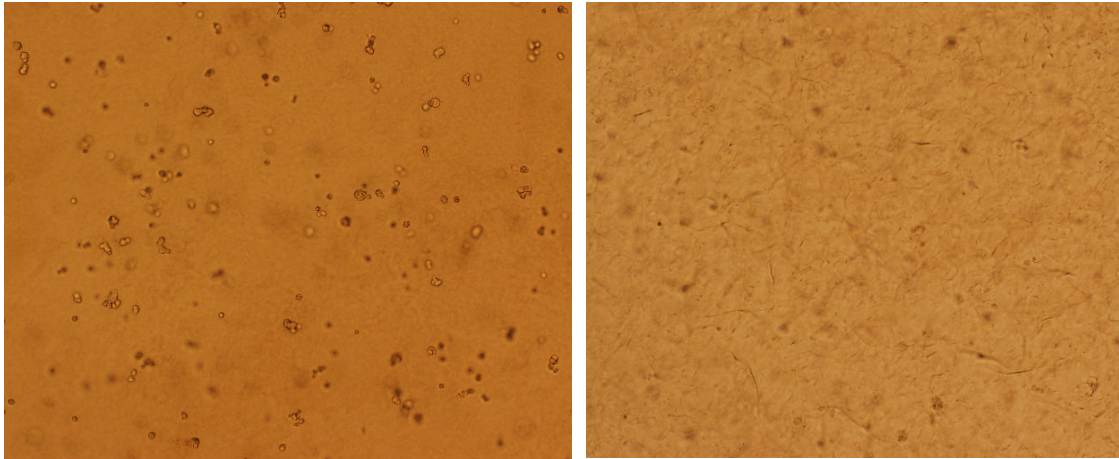
(a)

(b)

Figure 4.2 3mg/ml 200k/ml BR5 Microscopic Images at 4X Magnification (a) before DMA (b) after DMA.

#### 4.1.2 Microscopic Images Before and After Freezing

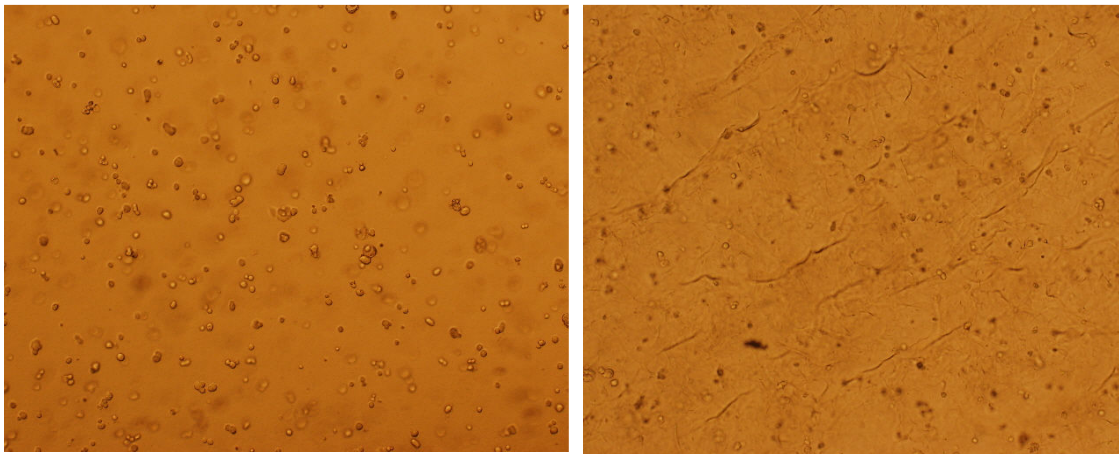
Figure 4.3, Figure 4.4, and Figure 4.5 show MCF-7 before and after freezing. These images are taken at different collagen and cell concentrations. Microstructural changes were noticed in the ETs after freeze/thaw. Similar changes were reported in Han et al (2005) and Miller (2006).



(a)

(b)

Figure 4.3 3mg/ml 200k/ml MCF-7 Microscopic Images at 4X Magnification of (a) unfrozen (b) frozen/thawed.

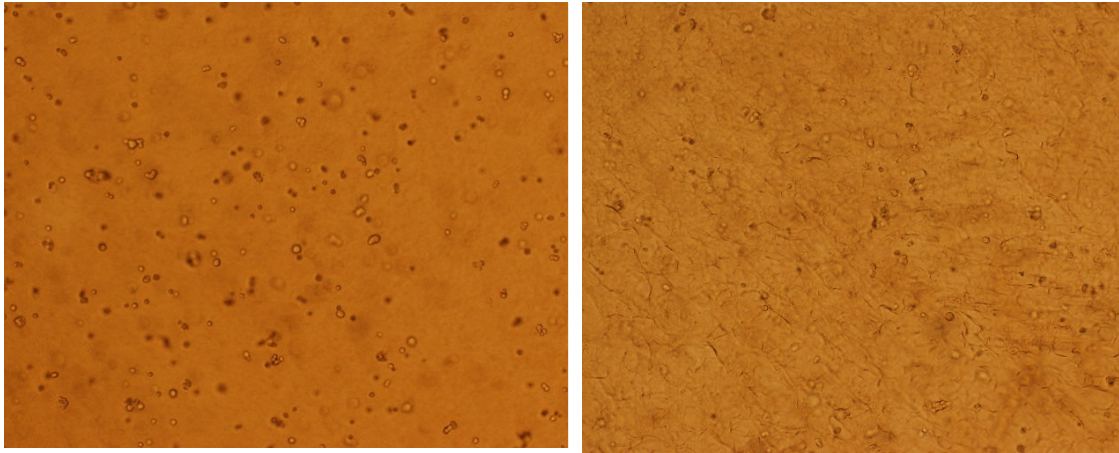


(a)

(b)

Figure 4.4 3mg/ml 400k/ml MCF-7 Microscopic Images at 4X Magnification of (a) unfrozen (b) frozen/thawed.



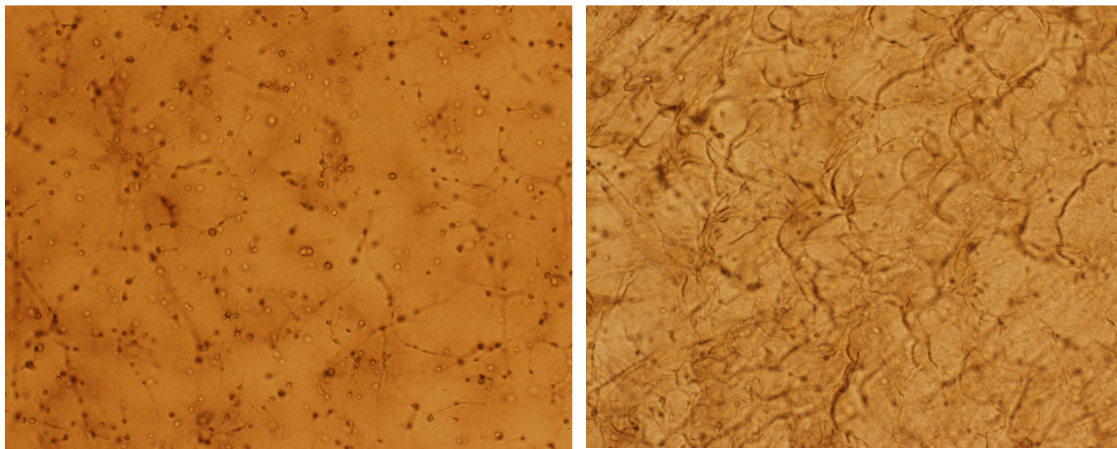


(a)

(b)

Figure 4.5 6mg/ml 200k/ml MCF-7 Microscopic Images at 4X Magnification of (a) unfrozen (b) frozen/thawed.

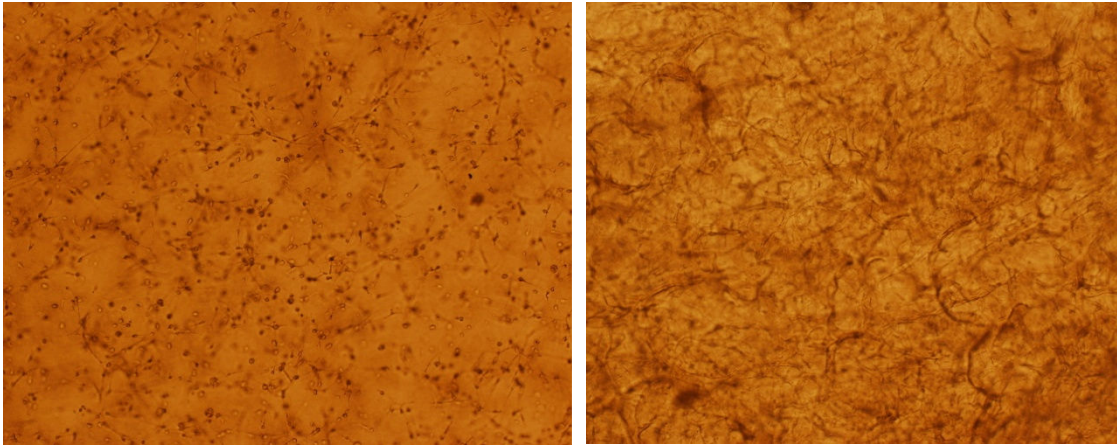
Figure 4.6, Figure 4.7, and Figure 4.8 show BR5 cells before and after freeze/thaw. These images were also taken at different cell and collagen concentrations. Similar structural changes are observed, but their extents are more intense than the ETs with MCF-7 cells.



(a)

(b)

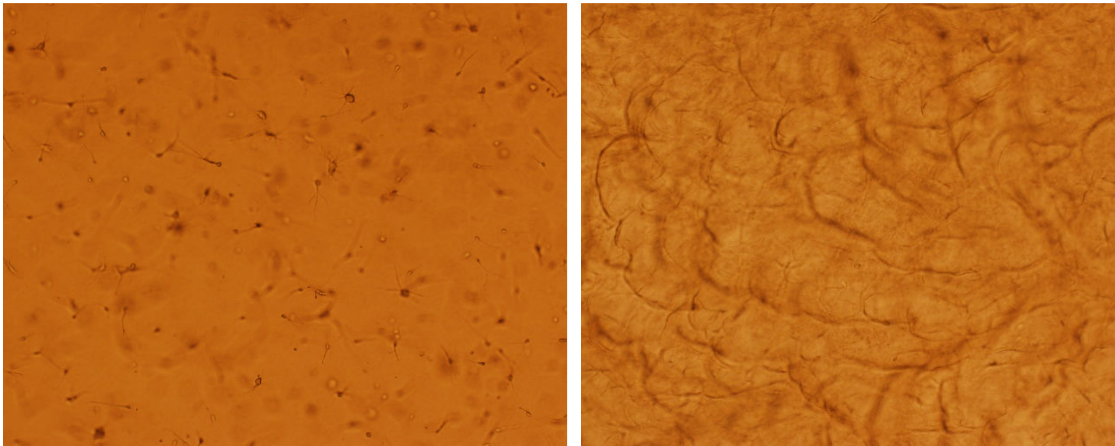
Figure 4.6 3mg/ml 200k/ml BR5 Microscopic Images at 4X Magnification of (a) unfrozen (b) frozen/thawed.



(a)

(b)

Figure 4.7 3mg/ml 400k/ml BR5 Microscopic Images at 4X Magnification of (a) unfrozen (b) frozen/thawed.



(a)

(b)

Figure 4.8 6mg/ml 200k/ml BR5 Microscopic Images at 4X Magnification of (a) unfrozen (b) frozen/thawed.

Figure 4.9 shows a sample ET with MCF-7 cells with 3mg/ml of collagen and 200,000 cells/ml after the freeze/thaw procedure. The left side of the ET is unfrozen and the right side of the ET was frozen and then thawed for 10 minutes.

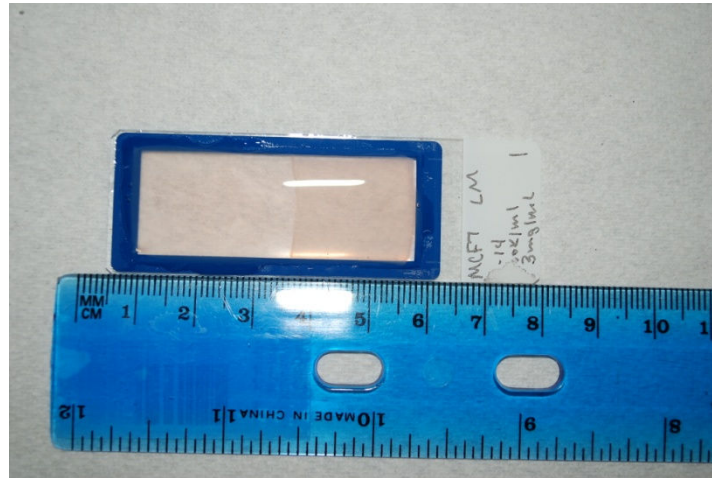


Figure 4.9 3mg/ml 200k/ml MCF7 Image after Freeze/Thaw Procedure.

#### 4.2 DMA Results

In this section the effect of four parameters on the viscoelastic properties of ET are described. The four parameters are 1) the effect of freeze/thaw, 2) cell concentration, 3) collagen concentration, and 4) cell type. The data obtained from the DMA is processed by a nonlinear regression analysis and the stress-strain response equations 2.1 and 2.2 to solve for two elastic parameters,  $E_0$  and  $E_1$ , and two viscous parameters,  $\eta_0$  and  $\eta_1$ . A sample creep-relaxation curve for MCF-7 unfrozen with 3mg/ml of collagen and 200,000 cells/ml can be seen in Figure 4.10. The stress for this ET is about 11.5Pa and the maximum strain observed is about 17%.

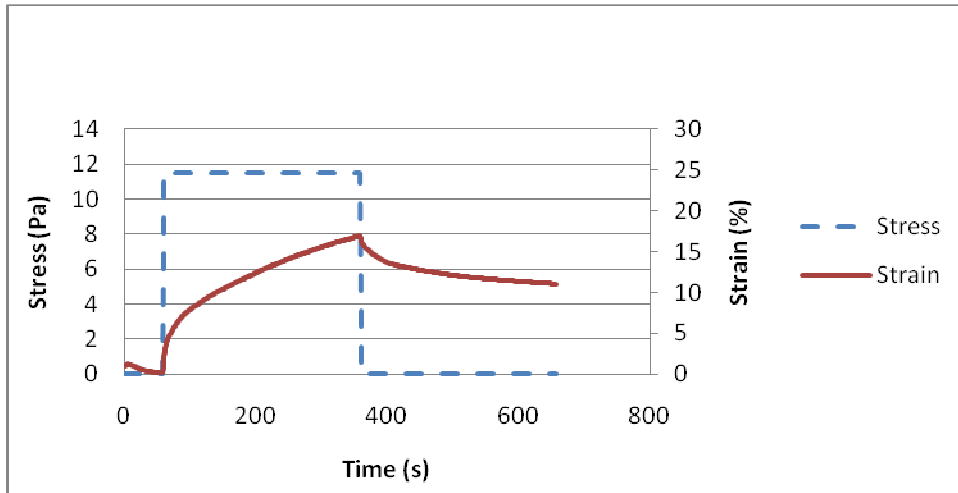


Figure 4.10 MCF-7 Creep-Relaxation Unfrozen.

A sample creep-relaxation curve for MCF-7 frozen/thawed with 3mg/ml of collagen and 200,000 cells/ml can be seen in Figure 4.11. The stress for this ET after freeze/thaw is about 11.5Pa and the maximum strain observed is about 14%.

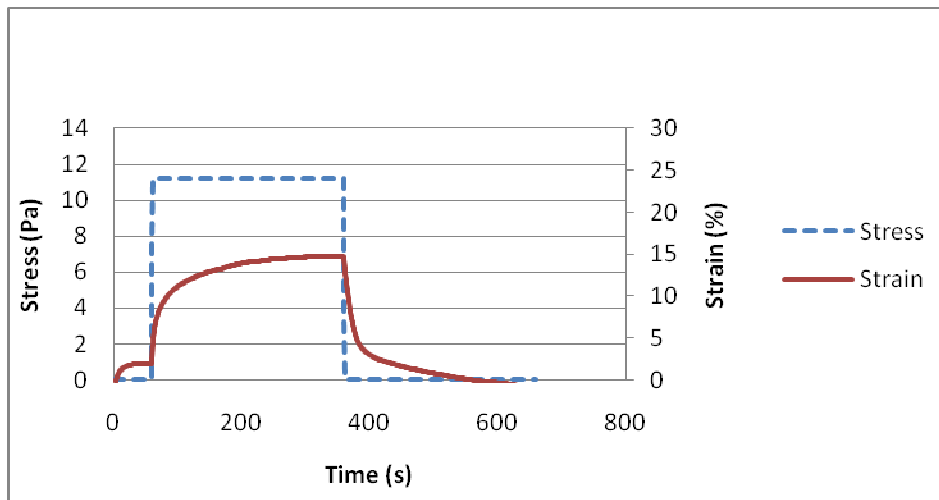


Figure 4.11 MCF-7 Creep-Relaxation Frozen/Thawed.

Table 4.1 and 4.2 show the mechanical properties results obtained for MCF-7 and BR5.

Table 4.1 MCF-7 Mechanical Properties Results.

200K/ML 3MG/ML				400K/ML 3MG/ML				200K/ML 6MG/ML			
AVERAGE	AVERAGE	SD	SD	AVERAGE	AVERAGE	SD	SD	AVERAGE	AVERAGE	SD	SD
UF	FT	UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
200.8	102.1	137.3	38.5	93.4	188.2	67.0	36.8	126.6	211.2	67.1	50.4
8940.1	8991.1	18.2	74.9	249.1	124.8	321.0	9.3	8956.0	8956.0	0.2	0.2
5437.0	400.7	7498.9	304.0	3930.4	1250.0	4087.3	239.0	386.7	515.9	267.5	151.6
546190.1	546190.1	0.0	0.0	546190.2	546190.2	0.0	0.0	546190.1	546190.1	0.0	0.0
19.5	4.3	18.9	2.9	2.9	2.5	0.6	0.4	3.3	2.6	1.7	0.1

Table 4.2 BR5 Mechanical Properties Results.

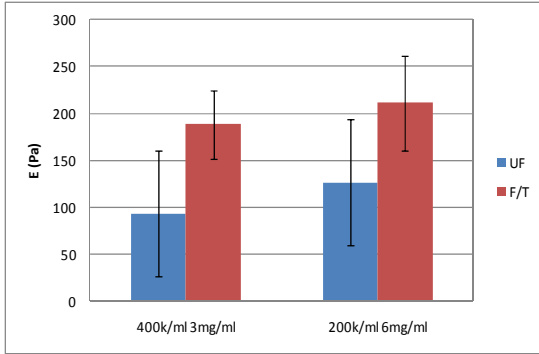
200K/ML 3MG/ML				400K/ML 3MG/ML				200K/ML 6MG/ML			
AVERAGE	AVERAGE	SD	SD	AVERAGE	AVERAGE	SD	SD	AVERAGE	AVERAGE	SD	SD
UF	FT	UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
47.8	109.3	58.9	53.5	280.8	239.1	80.8	208.7	506.6	716.2	367.8	852.3
8956.1	8956.1	0.0	0.0	23686.9	38466.6	25609.2	25684.1	14371.1	14896.5	22956.9	8022.9
158.1	246.2	155.3	99.0	1380.9	6893.6	660.9	8570.7	34228.9	25584.1	28610.8	37188.9
546190.1	546190.1	0.0	0.0	546191.6	546191.6	1.3	1.3	546191.3	546190.3	2.1	1.9
4.8	2.4	3.8	0.7	2.9	2.5	0.6	0.4	3.0	2.7	0.2	0.2

### 4.3 Effect of Freeze/Thaw on Viscoelastic Parameters

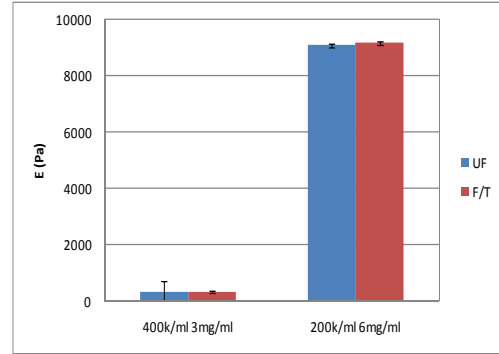
As discussed in section 4.2, the data gathered from the DMA was processed using equations 2.1 and 2.2 and solved for the viscoelastic parameters. The effects of freeze/thaw on these viscoelastic parameters for MCF-7 and BR5 are shown in Figure 4.12 through Figure 4.15.

Figure 4.12 (a) shows that the  $E_0$  for the HCELL ET (P=0.09) and the HCOLL ET (P=0.16) increases after freeze/thaw. This is also true in Figure 4.13 (a) for the HCOLL ET (P=0.72). It has been reported that freeze/thaw induce microstructural changes on the ETs including a decrease in tissue thickness, enlarged pore diameters, and thicker collagen fibril [9]. As for viscoelastic materials such as hydrogels, the equilibrium modulus,  $E_0$ , decreases with tissue thickness (i.e., water content) [15]. Since the freeze/thaw induced thickness decrease results from the efflux of interstitial fluid during freezing. [6], the equilibrium modulus should increase after freeze/thaw.

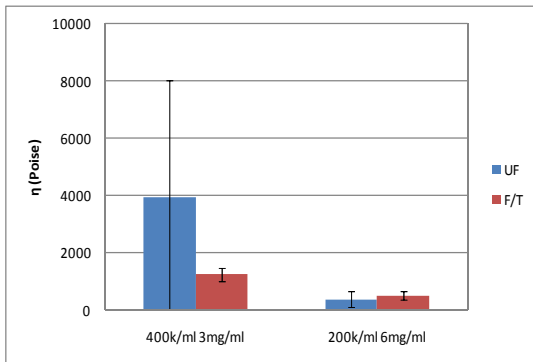
Figure 4.12 (b) shows that the instantaneous modulus increases after freeze/thaw for the HCOLL ET (P=0.78). Figure 4.13 (b) shows that  $E_1$  increases after freeze/thaw for the HCELL ET (P=0.52) and the HCOLL ET (P=0.97). It has also been reported that freeze/thaw induces enlarged pores on ETs which means that the pore diameter increases after freeze/thaw. As pore size increases, increase of tensile modulus and Young's modulus was reported elsewhere [16].



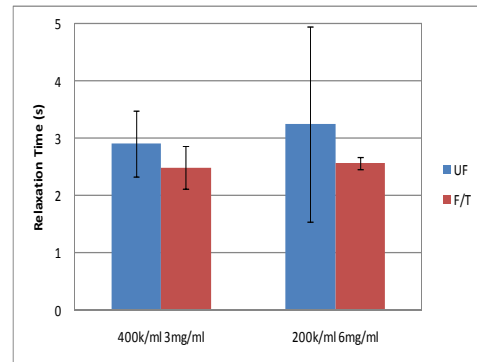
(a)



(b)



(c)



(d)

Figure 4.12 Effect of Freeze/Thaw on MCF-7 for (a) Equilibrium Modulus, (b) Instantaneous Modulus, (c) Apparent Viscosity, and (d) Relaxation Time.

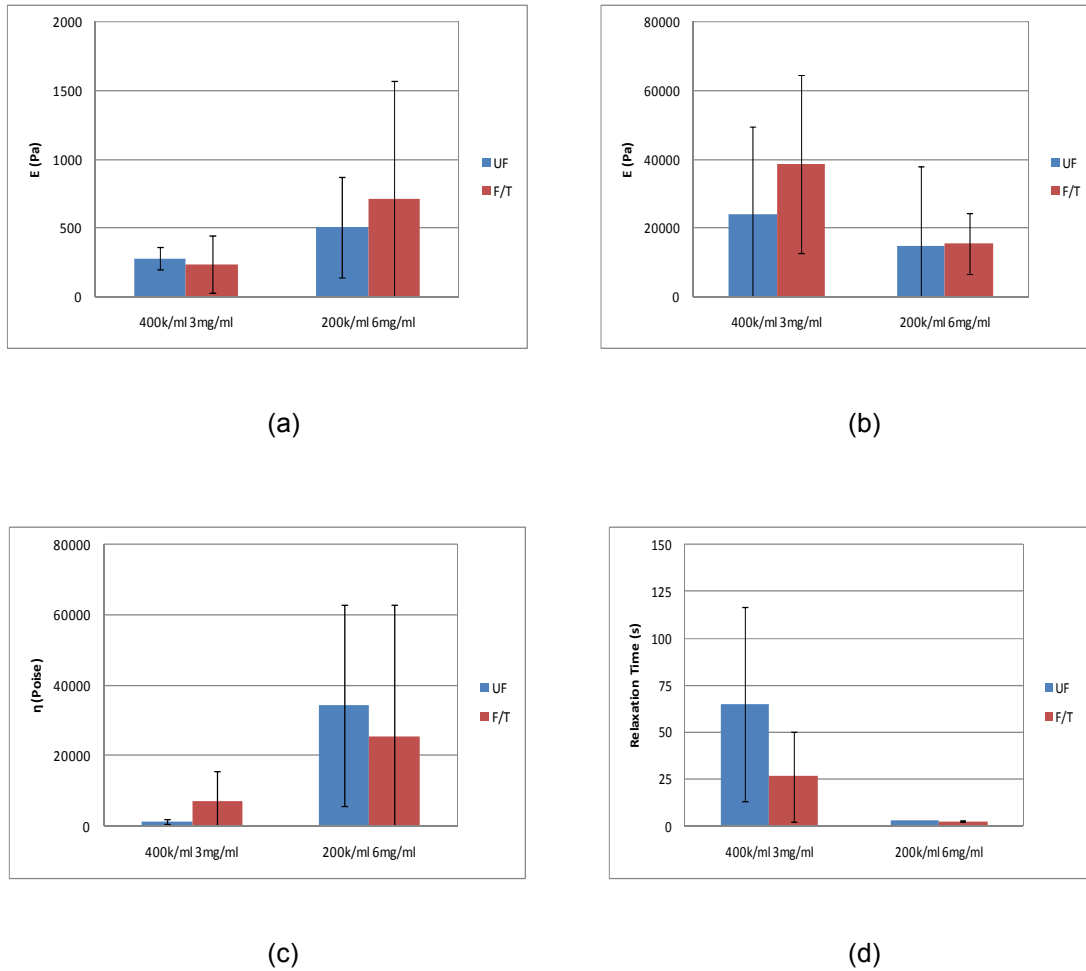


Figure 4.13 Effect of Freeze/Thaw on BR5 for (a) Equilibrium Modulus, (b) Instantaneous Modulus, (c) Apparent Viscosity, and (d) Relaxation Time.

Collagen fibril diameter increases after freeze/thaw. Freeze/thaw causes fibril area to decrease, mean void area to increase, and fibril diameter to increase. Mean void area increases means that there are less collagen fibrils present after the freeze/thaw process [9]. Also the collagen matrix of an unfrozen ET is dense and has an organized fibril network while an ET frozen to  $-20^{\circ}\text{C}$  is unorganized and has thicker fibril diameters [17].



#### 4.4 Effect of Cell Concentration on Viscoelastic Parameters

Figure 4.15 (a) shows that  $E_0$  for unfrozen ET ( $P=0.016$ ) increases as cell concentration increases. A higher cell concentration means that there is more compaction of the ECM which results in a denser matrix. An increase in cell concentration leads to an increase in mechanical properties. It was reported that the instantaneous modulus,  $E_0 + E_1$ , and the equilibrium modulus,  $E_0$ , increase with cell concentration [18].

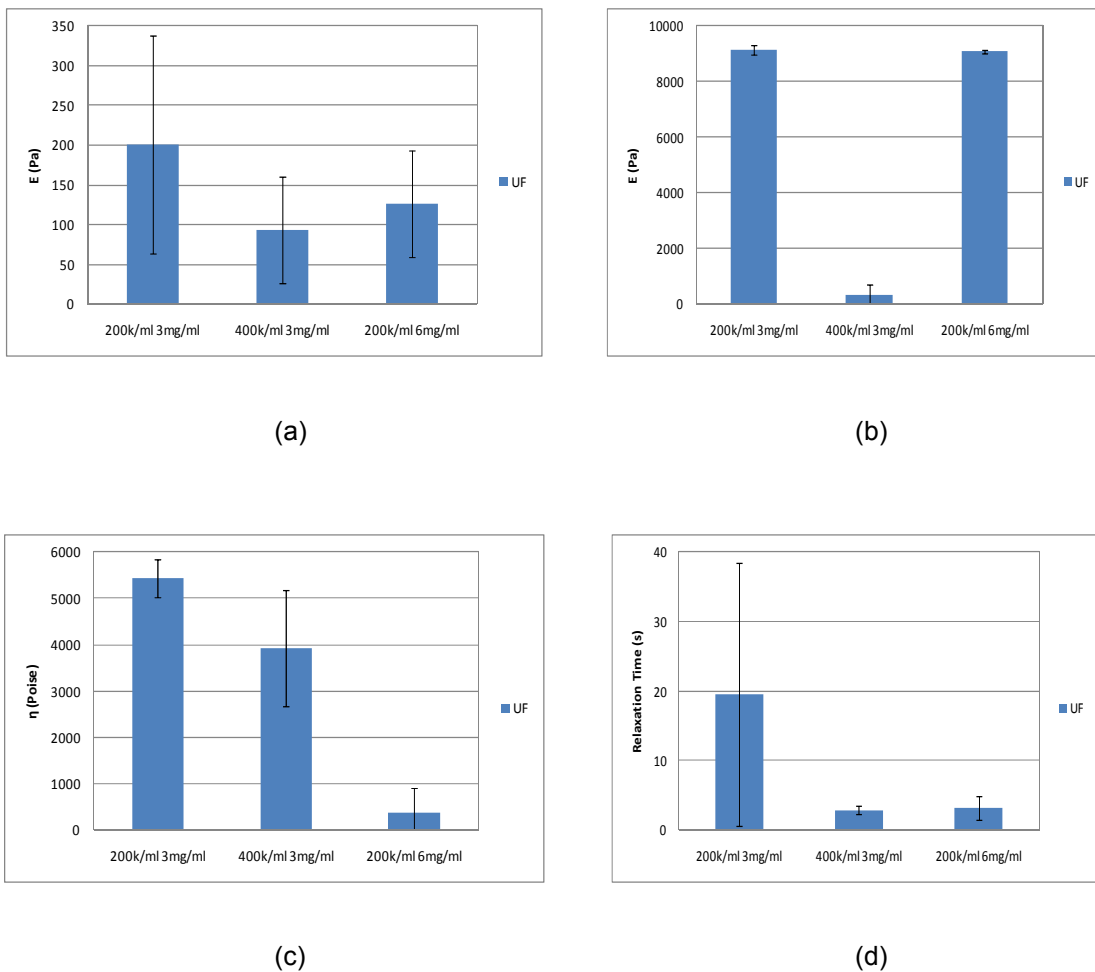
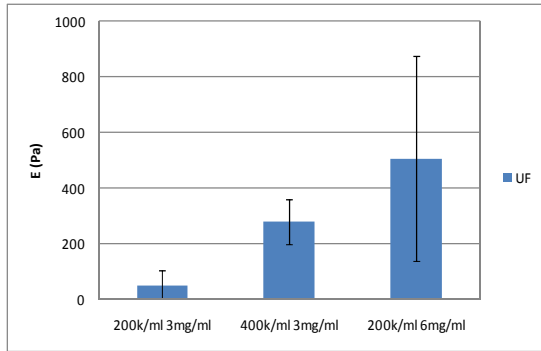
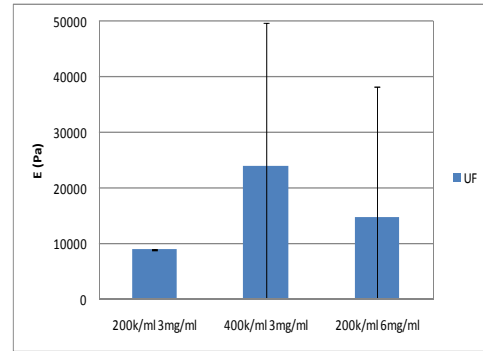


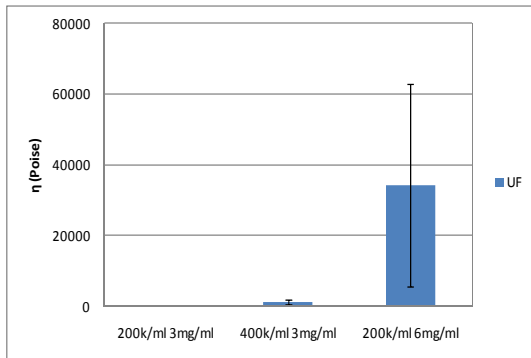
Figure 4.14 Effects of Cell and Collagen Concentration on MCF-7 for (a) Equilibrium Modulus, (b) Instantaneous Modulus, (c) Apparent Viscosity, and (d) Relaxation Time.



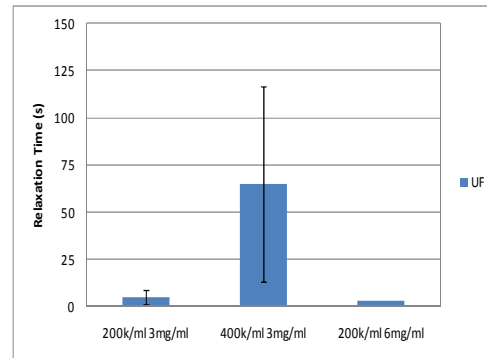
(a)



(b)



(c)



(d)

Figure 4.15 Effects of Cell and Collagen Concentration on BR5 for (a) Equilibrium Modulus, (b) Instantaneous Modulus, (c) Apparent Viscosity, and (d) Relaxation Time.

#### 4.5 Effect of Collagen Concentration on Viscoelastic Parameters

Appendix G shows that  $\eta_0$  remains the same as the collagen concentration increases for both unfrozen and frozen/thawed ( $P=0.35$  for both) ET for MCF-7. Figure 4.14 shows that  $\eta_1$  decreases for unfrozen ET as collagen concentration increases. Appendix G shows only a slight increase in  $\eta_0$  as collagen concentration increases ( $P=0.35$ ) for BR5. Figure 4.15 shows that  $\eta_1$

increases as collagen concentration increases for unfrozen ( $P=0.11$ ) ET. Figure 4.12 and Figure 4.13 show that both elastic parameters increase as the collagen concentration increases. Figure 4.12 (a) shows that  $E_0$  increases for frozen/thawed ET as the collagen concentration increases ( $P=0.29$ ). Figure 4.13 (b) shows that instantaneous modulus increases for unfrozen ET as the collagen concentration increases ( $P=0.20$ ). The effect of collagen concentration on viscoelastic parameters was reported that both viscous and elastic parameters increase as collagen concentration increases [19-22].

#### 4.6 Effect of Cell Type on Viscoelastic Parameters

Figure 4.15 shows that the elastic properties increase as cell concentration increases. Appendix G shows that only  $E_0$  for frozen/thawed ET increases as cell concentration increases for MCF-7 ( $P=0.049$ ). This may be due to BR5 is a fibroblast and MCF-7 is not. Fibroblasts compact the ET which causes a dense matrix and lower porosity. Fibroblasts condense a collagen matrix which comes directly from cellular traction. Tissues seeded with higher fibroblast cell concentration exhibit more rapid contraction and greater mechanical properties. It was reported that both instantaneous and equilibrium modulus increases with cell concentration due to contraction [18]. Figure 4.14 and Figure 4.15 show that the relaxation time of the Control ET for MCF-7 is greater than the Control for BR5.

## CHAPTER 5

### SUMMARY

As discussed, one of the most significant challenges of cryopreservation is to preserve the functional properties of tissues such as mechanical properties. Creep-relaxation tests were performed on engineered tissues to quantify changes of the viscoelastic properties of ETs before and after freeze/thaw. ETs with different tissue microstructures were studied including the cell concentration, collagen concentration, and cell type. The following observations were made:

- Instantaneous and equilibrium modulus increase as tissue thickness decreases which is induced by freeze/thaw.
- Instantaneous and equilibrium modulus increases with pore size and that pore size increases after freeze/thaw.
- Instantaneous and equilibrium modulus increases with cell concentration.
- Viscous and elastic properties increase with collagen concentration.
- Fibroblast BR5 cells have greater mechanical properties than MCF-7 cells due to contraction.

These observations indicate that cryopreservation outcome could be cell and tissue type dependent. The freezing process induces tissue thickness to decrease, pore size to increase, and the diameter of collagen fibrils to increase. It is observed that these changes due to freeze/thaw affect the viscoelastic properties of the ETs. Future research could include studying the mechanical properties of tissues using different testing methods with improved accuracy,

and comparing the mechanical properties obtained through creep-relaxation using the DMA. In addition, the effects of different freezing rates and conditions on the mechanical properties could be useful to design successful tissue cryopreservation methods.

APPENDIX A

STANDARD OPERATING PROCEDURE FOR CULTURING BR5

### **Equipments and reagents**

1. Glass pipettes
2. 75 cm<sup>2</sup> corning flasks
3. Advanced DMEM/F12 basal medium (Invitrogen, catalog no. 12634-010)
4. Fetal bovine serum (Invitrogen, catalog no. 16000-044)
5. L-glutamax (Invitrogen, catalog no. 35050-61)
6. Penicillin/Streptomycin (Invitrogen, catalog no. 15140-122)
7. Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen, catalog no. 14040-182)
8. Trypsin-EDTA (Invitrogen, catalog no. 25300-062)
9. DMSO (Sigma-Aldrich, product no. D2650)

### **Cell culture medium description and preparation**

Advanced DMEM/F12 basal medium supplemented with the following components:

- FBS, 10% (v/v)
  - L-glutamine, 2 mM
  - Penicillin, 100 units/mL
  - Streptomycin, 100 µg/MI
1. Warm a bottle of 500 mL Advanced DMEM/F12 basal medium.
  2. Thaw the following supplements:
    - FBS, 50 mL
    - L-glutamax, 5 mL
    - Penicillin/Streptomycin, 5 mL
  3. By using glass pipettes, transfer the supplements into the bottle of basal medium.
  4. Label the bottle with "BR5," date, and your initials.
  5. Store the bottle in the fridge at 4 °C.

### **Freezing medium description and preparation**

Advanced DMEM/F12 basal medium supplemented with the following components:

- FBS, 90% (v/v)
- L-glutamine, 2mM
- Penicillin, 100 units/mL
- Streptomycin, 100 µg/mL
- DMSO, 10% (v/v)

1. Warm the following reagents:
  - Supplemented cell culture medium
  - FBS, 25 mL
  - DMSO, 2 mL
1. Add 20 mL supplemented cell culture medium into a 50-mL microtube.
2. Transfer 17 mL FBS and 2 mL DMSO into the tube.
3. Label the tube with "BR5 freezing medium," date, and your initials.
4. Store the tube in the freezer at -20 °C.

### **Cell culturing method**

In general, culture cells in the supplemented medium, and incubate the cell culture at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

#### Common preliminary procedure

5. Pre-warm all necessary reagents (e.g., culture medium, DPBS, and trypsin).
6. Transfer all necessary equipments and reagents into the laminar flow hood. Wipe down the work surface of the hood with 70% ethanol. Wipe all bottles and equipments with 70% ethanol.

#### Thawing cryopreserved cells

1. Label a new flask with appropriate information (i.e., "BR5," date, and your initials).
2. Remove a vial of cells from the storage tank and immediately immerse it in a water bath at 37 °C; immerse only the vial up to the cap to prevent leakage. Agitate the vial in the warm water to thaw the cells as quickly as possible (no more than 2 minutes).
3. Wipe the vial with 70% ethanol and place it in the hood.
4. Transfer the cell suspension into a 15-mL microtube containing 9 mL of culture medium.
5. Centrifuge the tube at 2000 rpm for 4 minutes.
6. Resuspend the cell pellet in 20 mL of culture medium.
7. Transfer the cell suspension into the flask. Secure cap and incubate the flask.
8. Do not disturb the cell culture for 72 hours.

#### Cell sub-culturing

1. Aspirate old culture medium, and rinse cell sheet with 8 mL of DPBS twice.
2. Rinse cell sheet with 0.5 mL trypsin.
3. Add 2 mL trypsin to the flask, and tilt the flask to achieve uniform coverage.
4. Secure cap and incubate the flask.
5. Monitor the progress of cell dissociation at 5-10 min intervals.



6. When cells start to be released from the flask, cell dissociation can be hastened by giving the edge of the flask a sharp rap.
7. Add 8 mL medium, and gently disperse the cell suspension by repeated pipetting to break up cell clusters.
8. Depending on the chosen split ratio (1:2 or 1:4 is recommended), transfer an appropriate amount of aliquot into a new flask, and add the corresponding amount of fresh culture medium to the new flask to make up a total of 20 mL of aliquot. Label the new flask with "BR5," the split ratio (e.g., 1:2 or 1:4), date, and your initials.
9. Secure cap and incubate the new flask.
10. Collect the rest of the aliquot in the old flask into 15-mL microtube for experiment.

Note:

- BR5 has a population doubling time of approximately one week. Please change the cell culture medium and split the cell culture correspondingly.
- To achieve the highest cell densities, the culture medium should be changed every day as the culture approaches confluence.
- 1st-passage BR5 is capable of 20-25 population doublings, and thereafter the cells tend to lose their capability to proliferate.
- Please see the attached log sheet at the side of the cryotank for more information about the passage number of different BR5 cryotubes.

#### Cryopreserving cells

1. Centrifuge the tube containing  $\sim 1 \times 10^6$  cells at 2000 rpm for 4 minutes.
2. Discard the old medium, and resuspend the cell pellet in 1 mL of freezing medium.
3. Transfer the cell suspension into a cryotube.
4. Place the cryotube at 4 °C for 20-30 minutes.
5. Place the cryotube at -20 °C until it is frozen (several hours or overnight).
6. Finally, store the cryotube in liquid nitrogen storage tank.

#### **Protocol for removing mycoplasma**

1. Treat cells with 1:1000 dilution of Plasmocin (InvivoGen, catalog no. ant-mpt, stock conc.: 25 mg/ml) in culture medium for two weeks (change medium every 2-3 days, and split cells twice a week).
2. After one-week treatment, transfer  $\sim 5 \times 10^4$  cells onto a glass coverslip in a well (or chamber slide) with 1 mL of culture medium, and incubate it for 1-2 day.

3. Fix the cells with 3% HCHO (formaldehyde) for 10 min, and rinse the fixed sample with DPBS and then distilled H<sub>2</sub>O.
4. Mount the sample on a microslide with one drop of Vectashield mounting medium for DAPI fluorescence (Vector Lab, catalog no. H-1200).
5. Observe the sample under a microscope with DAPI filter. Mycoplasma contamination is indicated by small spots in the cytoplasm.
6. Repeat steps 1-5 after 2-week treatment.

### **References**

Rhee S, Jiang H, Ho CH, Grinnell F.

Microtubule function in fibroblast spreading is modulated according to the tension state of cell-matrix interactions.

Proc Natl Acad Sci U S A. 2007 Mar 27; 104(13): 5425-30.

Han B.

SOP for Cell Culture Media Preparation. July 28, 2006.

## APPENDIX B

### STANDARD OPERATING PROCEDURE FOR CULTURING MCF-7

### **Equipments and reagents**

1. Glass pipettes
2. Corning flasks
3. Advanced DMEM/F12 basal medium (Invitrogen, catalog no. 12634-010)
4. Fetal bovine serum (Invitrogen, catalog no. 16000-044)
5. L-glutamax (Invitrogen, catalog no. 35050-61)
6. Penicillin/Streptomycin (Invitrogen, catalog no. 15140-122)
7. Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen, catalog no. 14040-182)
8. Trypsin-EDTA (Invitrogen, catalog no. 25300-062)
9. DMSO (Sigma-Aldrich, product no. D2650)

### **Cell Culture medium description and preparation**

Advanced DMEM/F12 basal medium supplemented with the following components:

- FBS, 5% (v/v)
- L-glutamine, 2mM
- Penicillin, 100 units/mL
- Streptomycin, 100 µg/mL

1. Warm a bottle of 500 mL Advanced DMEM/F12 basal medium.
2. Thaw the following supplements:
  - FBS, 25 mL
  - L-glutamine, 5 mL
  - Penicillin/Streptomycin, 5 mL
3. By using glass pipettes, transfer the supplements into the bottle of basal medium.
4. Label the bottle with "MCF-7," date, and your initials.
5. Store the bottle in the fridge at 4°C.

### **Freezing medium description and preparation**

Advanced DMEM/F12 basal medium supplemented with the following components:

- FBS, 90% (v/v)
- L-glutamine, 2mM
- Penicillin, 100 units/mL
- Streptomycin, 100 µg/mL
- DMSO, 10% (v/v)

7. Warm the following reagents:
  - Supplemented cell culture medium
  - FBS, 25 mL
  - DMSO, 2 mL
1. Add 20 mL supplemented cell culture medium into a 50-mL microtube.
2. Transfer 17 mL FBS and 2 mL DMSO into the tube.
3. Label the tube with "MCF-7 freezing medium," date, and your initials.
4. Store the tube in the freezer at -20 °C.

### **Cell culturing method**

In general, culture cells in the supplemented medium, and incubate the cell culture at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

#### Common preliminary procedure

5. Pre-warm all necessary reagents (e.g., culture medium, DPBS, and trypsin).
6. Transfer all necessary equipments and reagents into the laminar flow hood. Wipe down the work surface of the hood with 70% ethanol. Wipe all bottles and equipments with 70% ethanol.

#### Thawing cryopreserved cells

9. Label a new flask with appropriate information (i.e., "MCF-7," date, and your initials).
10. Remove a vial of cells from the storage tank and immediately immerse it in a water bath at 37 °C; immerse only the vial up to the cap to prevent leakage. Agitate the vial in the warm water to thaw the cells as quickly as possible (no more than 2 minutes).
11. Wipe the vial with 70% ethanol and place it in the hood.
12. Transfer the cell suspension into a 15-mL microtube containing 9 mL of culture medium.
13. Centrifuge the tube at 1000 rpm for 3 minutes.
14. Resuspend the cell pellet in 9 mL of culture medium.
15. Transfer the cell suspension into the flask. Secure cap and incubate the flask.

#### Cell sub-culturing

11. Aspirate old culture medium, and rinse cell sheet with 8 mL of DPBS twice.
12. Rinse cell sheet with 0.5 mL trypsin.
13. Add 2 mL trypsin to the flask, and tilt the flask to achieve uniform coverage.
14. Secure cap and incubate the flask.
15. Monitor the progress of cell dissociation at 5-10 min intervals.
16. When cells start to be released from the flask, cell dissociation can be hastened by giving the edge of the flask a sharp rap.

17. Add 8 mL medium, and gently disperse the cell suspension by repeated pipetting to break up cell clusters.
18. Depending on the chosen split ratio (1:2 or 1:4 is recommended), transfer an appropriate amount of aliquot into a new flask, and add the corresponding amount of fresh culture medium to the new flask to make up a total of 10 mL of aliquot. Label the new flask with "MCF-7," the split ratio (e.g., 1:2 or 1:4), date, and your initials.
19. Secure cap and incubate the new flask.
20. Collect the rest of the aliquot in the old flask into 15-mL microtube for experiment.

**Note:**

- MCF-7 has a population doubling time of approximately two days. Please change the cell culture medium and split the cell culture correspondingly.

Cryopreserving cells

7. Centrifuge the tube containing  $\sim 1 \times 10^6$  cells at 1000 rpm for 3 minutes.
8. Discard the old medium, and resuspend the cell pellet in 1 mL of freezing medium.
9. Transfer the cell suspension into a cryotube.
10. Place the cryotube at 4 °C for 20-30 minutes.
11. Place the cryotube at -20 °C until it is frozen (several hours or overnight).
12. Finally, store the cryotube in liquid nitrogen storage tank.

**References**

Han B.

SOP for Cell Culture Media Preparation. July 28, 2006.

## APPENDIX C

### STANDARD OPERATING PROCEDURE FOR CASTING ENGINEERED TISSUE

		Final volume of collagen solution (in mL)									
		1	2	3	4	5	6	7	8	9	10
	Location	Amt. (μL)									
<b>10X MEM</b>	Fridge	100	200	300	400	500	600	700	800	900	1000
<b>Sterile 1.0 N NaOH</b>	Fridge	14	28	42	56	70	83	97	111	125	139
<b>Sterile dH2O</b>	Fridge	281	563	844	1125	1406	1688	1969	2250	2531	2813
<b>Rat Tail Collagen</b>	Fridge	605	1210	1815	2419	3024	3629	4234	4839	5444	6048
<b>1 M Hepes</b>	Fridge	0	0	0	0	0	0	0	0	0	0
<b>FBS</b>	Freezer	0	0	0	0	0	0	0	0	0	0
<b>P/S</b>	Freezer	0	0	0	0	0	0	0	0	0	0
<b>L- Glutamine</b>	Freezer	0	0	0	0	0	0	0	0	0	0
	<b>Total</b>	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000



## II. Preparation

- A. (2) 5 mL pipettes
- B. 1000, mL, 250 mL and 20 mL pipette tips
- C. Chamber slides
- D. 15 mL centrifuge tube
- E. Cell culture medium (cell-type dependent)
- F. Type I High Concentration Collagen
- G. Container with ice cubes

## III. Procedure: for 2 mL sample

NOTE: Keep NaOH and dH<sub>2</sub>O on ice until use; carry out procedures in hood when possible

Warm bath to 37 deg. C; put on gloves; spray with ethanol

A. Warm MEM and Hepes to ~ 37 deg. in waterbath

1. Thaw P/S, L-Glutamine, and FBS in waterbath; WATCH MOUTH OF TUBE.

DON'T CONTAMINATE

B. Spray inside bottom of hood with ethanol and wipe

C. Prep pipette knobs to correct sucking amount

D. Retrieve tupperware bowl, spray with ethanol and wipe, put ice in it, put it in hood, put 15 mL centrifuge tube in it

E. Retrieve, spray with ethanol, and wipe down NaOH and dH<sub>2</sub>O and place them in the bowl of ice in hood

1. Retrieve, wipe down, and spray with ethanol the Hepes, P/S, FBS, and L-Glut and place in hood

F. Retrieve, wipe down, spray with ethanol, and wipe down MEM, then add to the chilled centrifuge tube (in hood) with 250 mL pipette tip

G. Add sterile, ice cold NaOH to MEM (in hood) with 250 mL pipette tip

H. Add Hepes, P/S, L-Glutamine, and FBS

I. Add sterile, ice cold dH<sub>2</sub>O (in hood) with 1000 mL pipette tip

J. Add solution to washed cell pellets (in 15 mL centrifuge tube)

K. Mix the contents of the tube by quickly pipetting several times within one minute (avoid bubbles) and hold in ice (practice needed); use 5 mL pipette.

L. Prepare the chamber slide or petri dish (retrieve, mark, and open), new 5 mL pipette, and pipette knob while the tube is on ice

M. Re-spray hands with ethanol, retrieve, spray with ethanol and wipe down collagen. Put on ice in hood.

N. Add collagen (avoid bubbles) with 1000 mL pipette tip and mix using 5 mL pipette from J.

O. Dump the mixture into chamber slide or petri dish using the same 5 mL pipette

P. Place the solution in the incubator and allow to gel at 37°C for 30 min.

Q. While in incubator, refill ice tray and put in freezer; retrieve, spray with ethanol, wipe down, and put PBS in hood

Put MEM, collagen, NaOH, and dH<sub>2</sub>O back in fridge

R. When gel is ready, add 2 mL culture media to cover the whole gel with 2 mm thickness (for chamber slides)

S. Place in incubator until experiment

NOTE: Check culture medium thickness periodically to avoid gel dehydration

Also, be sure to remove culture media from surface of gel before freezing, fixing, etc.

NOTE: Keep NaOH and dH<sub>2</sub>O on ice until use; carry out procedures in hood when possible.

APPENDIX D

STANDARD OPERATING PROCEDURE FOR DYNAMIC MECHANICAL ANALYSIS

## Equipment

1. Gel
2. DMA
3. Nitrogen Gas
4. Liquid Nitrogen
5. Wrench
6. 70 % ethanol

## Set up

1. Fill dewer with Liquid Nitrogen. (The dewer is the small storage tank for the liquid nitrogen) Connect hose from dewer to Nitrogen tank to the line labeled Liquid using a wrench. Open the valve on the Liquid line.
2. On the computer open TA Instrument Explorer, Q800-0996. Click **Control, GCA Fill**. The dewer will begin to fill with liquid nitrogen and will stop when it is full. When it is done close the valve to the liquid nitrogen. Once the dewer is filled, tests must be performed within 2-3 days.
3. Turn on Nitrogen Gas above 60 psi (The nitrogen tank should be hooked up to the DMA)
4. On DMA touch screen touch **Open Furnace, Apply. Float, Apply**.
5. When using the Compression Clamp, lift the top plate with your hands and touch **Lock, Apply**. As Seen in Figures 1 & 2.
6. Place gel in cup. (Unless gel was made in the cup already). The cup screws on and off.
7. \*\*\*Touch **Float, Apply** and lower top plate until it barely touches the gel surface. Then **Lock, Apply** and **Close Furnace, Apply**.

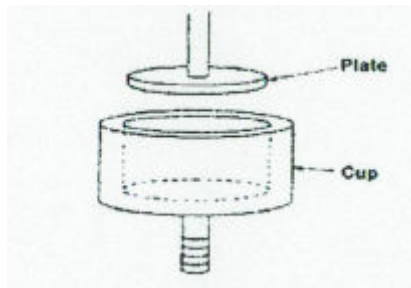


Figure 1 Cup<sup>2</sup>



**Figure 2 Compression Clamp- fixed flat surface and an oscillating plate applies force.<sup>1</sup>**

Stability (Q800-0996):

1. Tools, Instrument Preferences, DMA
2. Stabilization cycles: choose between 0-4, Normal is 4
3. Stress/Force Accuracy tolerance: choose between 5-50, Normal is 5%
4. Check Collect only when stable

There are three standard types of testing including: Set Temperature, Temperature Scan, and Creep-Recovery. An example set up for each test can be seen below.

### **Test 1- Set Temperature**

Summary Tab

Mode: DMA Multi-Frequency Strain

Test: Custom

Clamp: Compression

Shape: round

Dimensions: For disc 2mm x 19.5 mm (Thickness x Diameter)

Sample Name: choose file name

Data File: choose location (must be a shared file) C Drive , TA, Data, DMA, create your own folder

Click **Apply**

#### Procedure

Test: Custom

Amplitude: 5  $\mu\text{m}$ -50  $\mu\text{m}$ , Example 15  $\mu\text{m}$

Preload Force: choose between .001N to 0.1 N, Example .0001N

Uncheck Force Track when testing above freezing

#### Editor (Example set up)

1. Equilibrate at 20° C
  2. Data Storage On
  3. Isothermal for 5 minutes
  4. Frequency Sweep
- Repeat Segment 4 for 1 time

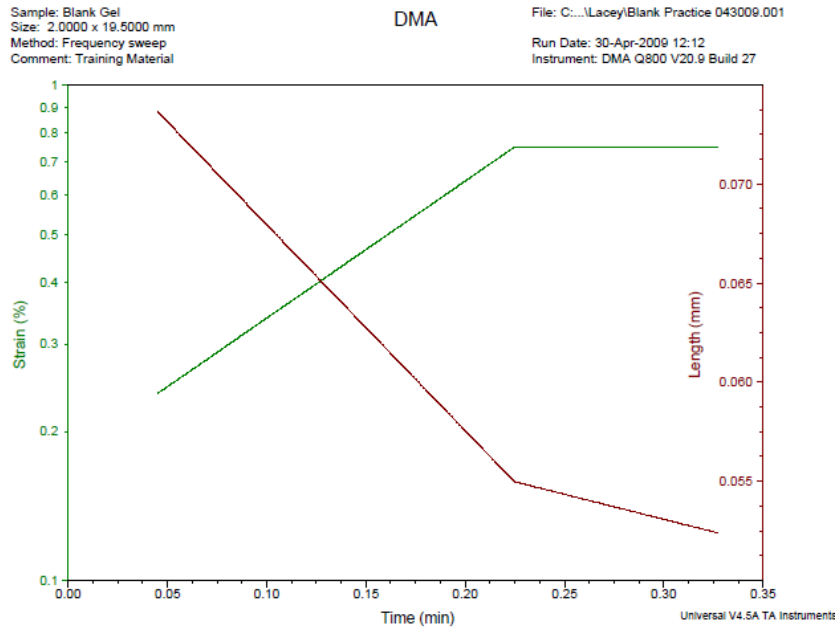
Frequency table Tab Select: Single, Log, Linear, or Discrete. Example: Single 5 Hz

Click **Apply**

Click **Play**

If the storage and loss modulus values are negative then there is an error and the test must be stopped.

Progress of the test can be seen on the right hand side of the screen showing the signal, value, and a plot.



**Figure 3 Time versus strain and length.**

**Test 2- Temperature Scan**

Summary

Mode: DMA Multi-frequency Strain

Test: Custom

Clamp: Compression

Shape: round

Dimensions: 2mm x 19.5 mm

Sample Name:

Data File: must be a shared file, C, TA, Data, DMA, create your folder

Click **Apply**

#### Procedure

Test: Temp Ramp/Freq Sweep

Amplitude: 5  $\mu\text{m}$ -50  $\mu\text{m}$ , Example 5  $\mu\text{m}$

Preload Force: choose between .001N to 0.1 N. Example .03N

Force track: (if below freezing) 115%-150%. Example 125%

Advanced: Option to change data sampling interval

Post Test: Option to open furnace, change clamp to up, float, or lock, or GCA auto fill

#### Example Set Up

Start Temp: Check use current

Soak Time: 5 minutes

Final Temperature:  $-40^{\circ}\text{C}$

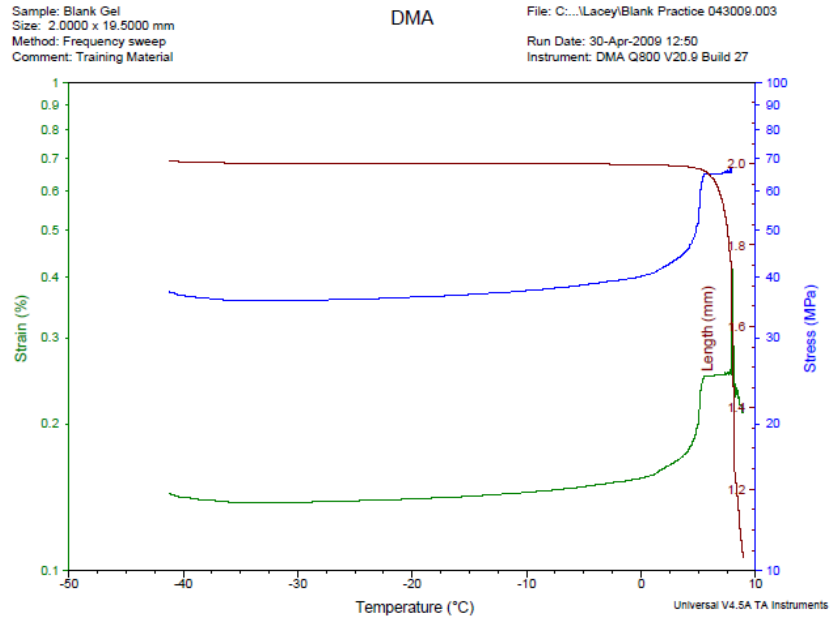
Ramp Rate:  $1^{\circ}\text{C}/\text{min}$

#### Frequency Table

Select: Single, Log, Linear, or Discrete. Example: Single 5 Hz

Click **Apply**

Click **Run**



**Figure 4 Temperature versus strain, stress, and length**

**Test 3- Creep-Recovery**

Summary

Mode: DMA Creep

Test: Custom

Clamp: Compression

Shape: round

Dimensions: 2mm x 19.5 mm

Sample Name:

Data File: must be a shared file, C, TA, Data, DMA, create your folder

Procedure



Test: Creep

Preload Force: choose between .0001N to 0.1 N, Example .0001N

Stress: choose between 5-50 MPa, Example .005MPa

Advanced: Option to change data sampling interval

Post Test: Option to open furnace, change clamp to up, float, or lock, or GCA auto fill

Example set up

Isothermal Temperature: 20° C

Soak Time: 5 minutes

Creep Time: 5 minutes

Recovery Time: 10 minutes

Click **Apply**

Click **Play**

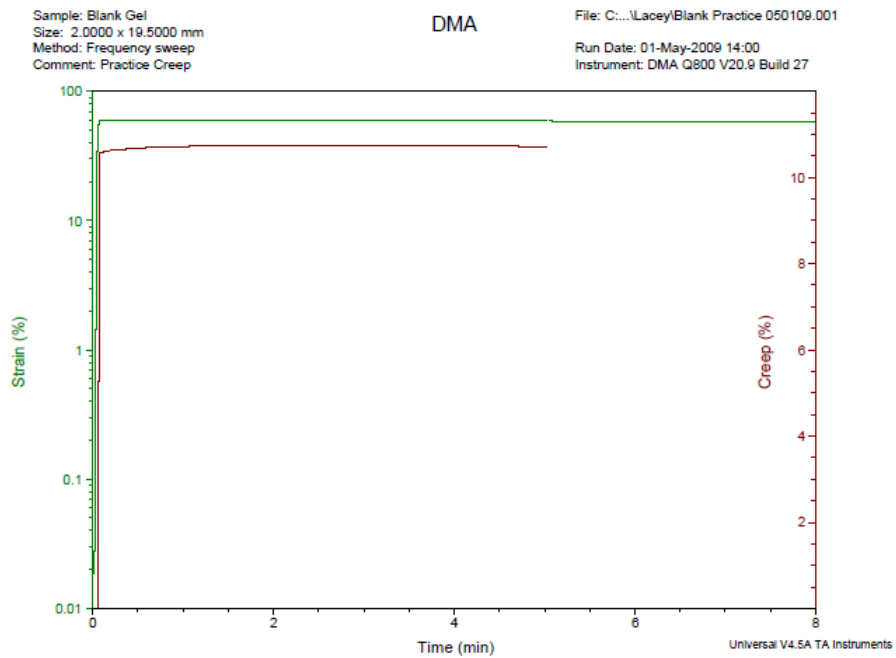


Figure 5 Temperature versus Creep Compliance, Creep, and Recoverable Compliance

### Viewing Data

To view data click **Launch Win UA** . Click **File, Open** and choose file.

In the Data File Information window click on **Signals**.

In the Signals window choose x- and y-axis. You can choose up to four y- axis. Type of scale can be chosen to be normal, log, inverse, etc. Click **Ok** on both windows. A plot of your data will open (Universal Analysis 2000).

To change your axis click **Graph, Signals**. Right click on y-axis to change line type, grid type, color, and symbols. Right click on x-axis to do manual rescaling.

There are many options in the Universal Analysis 2000 window such as spreadsheets, tables, reports, and converting the file to .pdf. To open a Microsoft Excel Spreadsheet click **View, Data Table, Spreadsheet**, select **all data point** and **Ok**.

### Shut Down

When finished testing remove cup and top plate. Discard gel and clean cup and plate with 70% ethanol. Screw cup and top plate back onto clamp. Lock clamp in open position and close furnace. Turn off Nitrogen gas. Make sure the liquid nitrogen tank is also closed.

### Calibration

#### **Clamp Calibration:**

1. when changing clamps
2. if clamp does not float properly
3. suspect incorrect sample length measurements
4. improper clamping
5. after loading new instrument software

#### **Clamp Mass**

Follow the steps below:

1. Clear the clamp area on the instrument. Do not mount a sample. If using a submersion clamp skip to step 3.
2. Press the **FURNACE** key to close the DMA furnace. Do not close furnace when submersion clamp is installed.

3. Press the **CALIBRATE** button to begin the clamp mass calibration step. The furnace will open when the clamp mass calibration has completed. (It normally takes approximately five minutes to complete the clamp mass calibration.)
4. Select the **Next** button to proceed when the calibration step is complete.

#### **Clamp Zero**

Follow the steps below:

1. Clear the clamp area on the instrument. (Do not mount a sample.) If you are using a submersion type clamp, skip to step 3.
2. Press the **FURNACE** key to close the DMA furnace. (Do not close the furnace when submersion type clamp is installed.)
3. Press the **Calibrate** button to begin the clamp zero calibration. The clamp faces will be pressed together to determine the zero length point.
4. Select the **Next** button to proceed when the calibration step is complete.

#### **Clamp Zero with Offset Gauge**

Follow the steps below:

For Film/Fiber Tension Clamps

1. Measure the offset gauge, found in the accessory kit, with calipers to determine its length.
2. Enter the length of the offset gauge (range of 5 to 30 mm) in the appropriate field.
3. Mount the offset gauge in the fixed clamp. If you are using a submersion type clamp, skip to step 4.
4. Press the **FURNACE** key to close the DMA furnace. (Do not close the furnace when a submersion type clamp is installed.)
5. Press the **Calibrate** button to begin the calibration step. The zero length point for the sample is determined to be the underside of the fixed clamp. The furnace will open when the clamp zero calibration has been completed.
6. Remove the offset gauge when the furnace opens.
7. Press **Next** button to proceed to the next window.

For Compression or Penetration Clamps

1. If your clamp needs a gauge block, enter its size in the gauge block length field. (These clamps normally do not use a gauge block, so this entry can be left unchanged at 0.0.) If you are using a submersion type clamp, skip to step 3.
2. Press the **FURNACE** key to close the DMA furnace. (Do not close the furnace when a submersion type clamp is installed.)
3. Select **Calibrate**. The clamp faces will be pressed together to determine the zero length point. The furnace will open when the clamp zero calibration has completed.
4. Press **Next** to proceed to the next window.

#### **Clamp Compliance (with Sample)**

1. Measure the precision steel compliance sample, provided in the accessory kit, with calipers to determine its thickness and width.
2. Enter the compliance sample dimensions (in this order - length, width, and thickness). Note: For Dual cantilever clamps use length = 35 mm.
3. Mount the compliance sample and measure the sample length. Use a torque of 8 to 10 in-lbs for dual/single cantilever clamps. Use a torque of 2 to 3 in-lbs for film tension clamps when mounting the sample. If you are using a submersion type clamp, skip to step 5.
4. Press the **FURNACE** key to close the DMA furnace. (Do not close the furnace when a submersion type clamp is installed.)
5. Select the **Calibrate** button. The furnace will open when the compliance calibration has completed.
6. Press **Next** to proceed to the next window.

### **Clamp Compliance (without steel sample)**

For Compression or Penetration Clamps

1. Press the **FURNACE** key to close the DMA furnace.
2. Select the **Calibrate** button to calibrate the clamp compliance.
3. Press **Next** to proceed to the next window.

### **Position Calibration:**

1. When the DMA is moved or at least once a month
2. if the probe will not float
3. If the instrument is turned off without locking the drive shaft first

### **Instrument Calibration:**

1. When instrument is moved
2. If the force is not linear over the entire traveling distance of the movable clamp
3. If modulus and tan delta have unusual responses to changes in frequency
4. if the feed hose for the cool air or GCA is removed or installed
5. regular (monthly) bases

### **Electronics Calibration:**

1. Automatically removes the air to lock the drive shaft and applies a static force to the motor as a calibration reference.
2. Performed at least once a month.

Follow the steps below:

1. Select **Calibrate/Instrument/Electronics** from the menu.
2. Remove both the fixed and moveable clamps.
3. Press the **DRIVE** key until the drive shaft can move up and down freely.
4. Install the shipping bracket. See [Installing/Removing the Shipping Bracket](#) for instructions in the Help Menu.
5. Press the **FURNACE** button to close the furnace.
6. Click the **Calibrate** button.
7. Remove the shipping bracket when the calibration is complete.
8. Select **Next** to view the [Calibration Report](#).
9. Perform the [Force Calibration](#) procedure after the Electronics calibration has been completed.

### **Force Calibration:**

1. Used to adjust the force exerted by the clamp on the sample and the force registered by the instrument as the experiment proceeds.

Follow the steps below to perform the force/balance calibration:

1. Step 1. Select **Calibrate/Instrument/Force** from the menu.
2. Remove both the fixed and moveable clamps.
3. Press the **FURNACE** key to close the DMA furnace.
4. Select **Calibrate**. The instrument will be calibrated automatically. The furnace will open when the calibration has completed. (It normally takes approximately five minutes to complete the force/balance calibration.)
5. Select the **Next** button to proceed to the [force/weight](#) calibration.
6. Step 2. Make sure that you have just completed the force/balance calibration step 1, as directed.
7. Place the 100-gram weight on top of the drive shaft dovetail. Make sure that the weight is centered on the dovetail and that the thermocouples do not touch the weight.
8. Press the **FURNACE** key to close the DMA furnace.

9. Select **Calibration**. The instrument will be calibrated automatically. The furnace will open when the calibration has completed. (It normally takes approximately five minutes to complete the force/balance calibration.)
10. Remove the weight.
11. Click the **Next** button to view the Calibration Report summary.
12. Perform the Dynamic calibration after the force/weight calibration has been completed.

## **References**

1. TA Instruments Q Series Thermal Analyzers
2. TA Instruments Q800 Help Menu
3. Dynamic Mechanical Analysis A Practical Introduction, Menard, CRC Press LLC, 1999

APPENDIX E

ORIGINAL VISCOELASTIC PARAMETERS DATA

	BR5		t=0 to 50							
	200k/ml 3mg/ml									
	1		2		3		Average	Average	Stand Dev	Stand Dev
	UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
E_o	10.93	169.49	16.69	90.92	115.77	67.37	47.79	109.26	58.94	53.47
E_1	8956.11	8956.09	8956.06	8956.05	8956.07	8956.06	8956.08	8956.07	0.03	0.02
$\eta_1$	100.54	322.94	39.81	281.32	333.96	134.40	158.10	246.22	155.30	99.05
$\eta_0$	546190.08	546190.08	546190.08	546190.08	546190.08	546190.08	546190.08	546190.08	0.00	0.00
R^2	0.97	0.86	0.92	0.86	0.86	0.87	0.91	0.86	0.06	0.01
	400k/ml 3mg/ml									
	1		2		3		Average	Average	Stand Dev	Stand Dev
	UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
E_o	265.13	0.04	368.26	332.34	208.954	384.90	280.78	239.09	80.80	208.69
E_1	8993.57	53333.00	53257.67	53257.53	8809.544	8809.16	23686.93	38466.56	25609.18	25684.09
$\eta_1$	1210.10	16775.00	2110.46	1478.29	822.2479	2427.42	1380.94	6893.57	660.88	8570.72
$\eta_0$	546190.10	546190.10	546192.42	546192.42	5.46E+05	546192.42	546191.65	546191.65	1.34	1.34
R^2	0.83	0.60	0.86	0.89	0.89	0.93	0.86	0.81	0.03	0.18
	200k/ml 6mg/ml									
	1		2		3		Average	Average	Stand Dev	Stand Dev
	UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
E_o	868.38	142.18	518.50	1695.50	133.00	310.78	506.63	716.15	367.83	852.32
E_1	40861.19	22851.78	1977.85	6807.68	274.30	15030.00	14371.11	14896.49	22956.89	8022.88
$\eta_1$	52953.84	280.17	48437.93	68282.66	1295.02	8189.46	34228.93	25584.10	28610.84	37188.91
$\eta_0$	546192.56	546192.47	546192.51	546189.45	546188.95	546188.95	546191.34	546190.29	2.07	1.91
R^2	0.95	0.86	0.89	0.64	0.91	0.99	0.92	0.83	0.03	0.18

MCF7		t=0 to 50									
		200k/ml 3mg/ml									
		1		2		3		Average		Stand Dev	
		UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
E_o		144.37	139.54	357.24	104.03	100.66	62.71	200.76	102.09	137.27	38.45
E_1		8943.44	8940.07	8920.51	9077.05	8956.36	8956.11	8940.10	8991.08	18.15	74.89
$\eta_1$		1685.84	193.77	14071.27	749.73	553.78	258.58	5436.96	400.69	7498.93	304.01
$\eta_0$		546190.07	546190.07	546190.08	546190.08	546190.08	546190.08	546190.08	546190.08	0.01	0.01
R^2		0.96	0.77	0.90	0.92	0.83	0.88	0.90	0.86	0.07	0.08
		400k/ml 3mg/ml									
		1		2		3		Average		Stand Dev	
		UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
E_o		167.06	171.06	77.01	163.04	36.16	230.47	93.41	188.19	66.97	36.83
E_1		618.32	123.39	36.24	116.27	92.68	134.73	249.08	124.80	321.02	9.31
$\eta_1$		1842.36	1151.62	1308.96	1522.48	8639.99	1075.92	3930.44	1250.01	4087.30	238.98
$\eta_0$		546190.23	546190.23	546190.23	546190.23	546190.24	546190.23	546190.23	546190.23	0.01	0.00
R^2		0.97	0.99	0.96	0.99	0.93	0.95	0.95	0.98	0.02	0.02
		200k/ml 6mg/ml									
		1		2		3		Average		Stand Dev	
		UF	FT	UF	FT	UF	FT	UF	FT	UF	FT
E_o		118.82	225.64	197.21	252.88	63.77	155.20	126.60	211.24	67.06	50.41
E_1		8956.12	8956.19	8955.93	8955.95	8955.82	8955.84	8955.95	8955.99	0.15	0.18
$\eta_1$		331.59	641.68	677.50	558.43	151.05	347.64	386.71	515.92	267.52	151.56
$\eta_0$		546190.08	546190.08	546190.08	546190.08	546190.08	546190.08	546190.08	546190.08	0.00	0.00
R^2		0.83	0.86	0.84	0.85	0.84	0.85	0.84	0.85	0.01	0.01

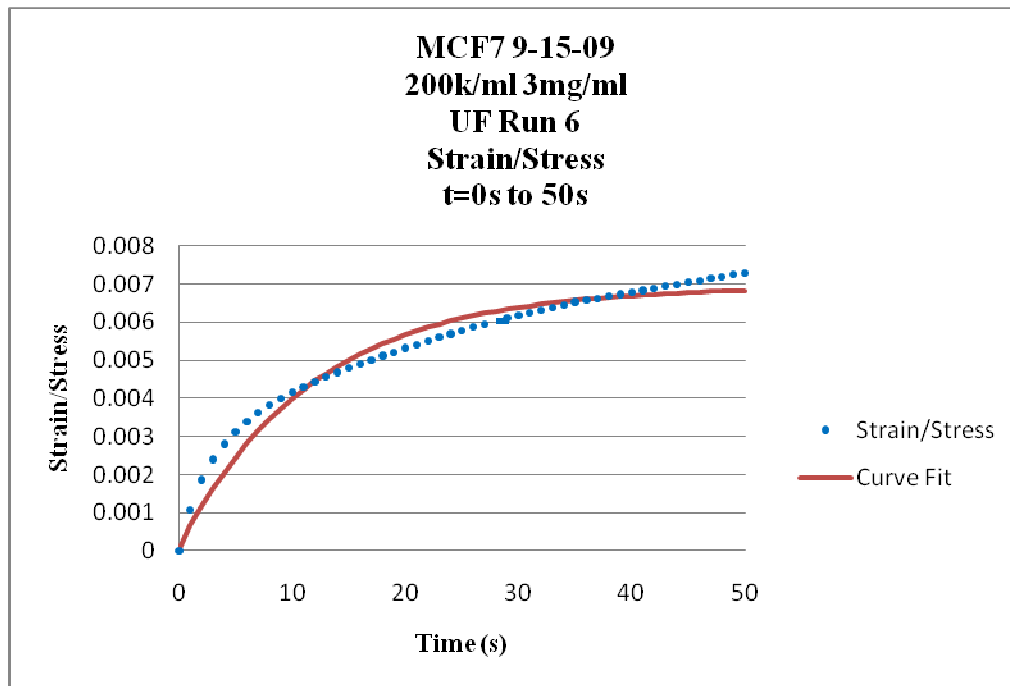


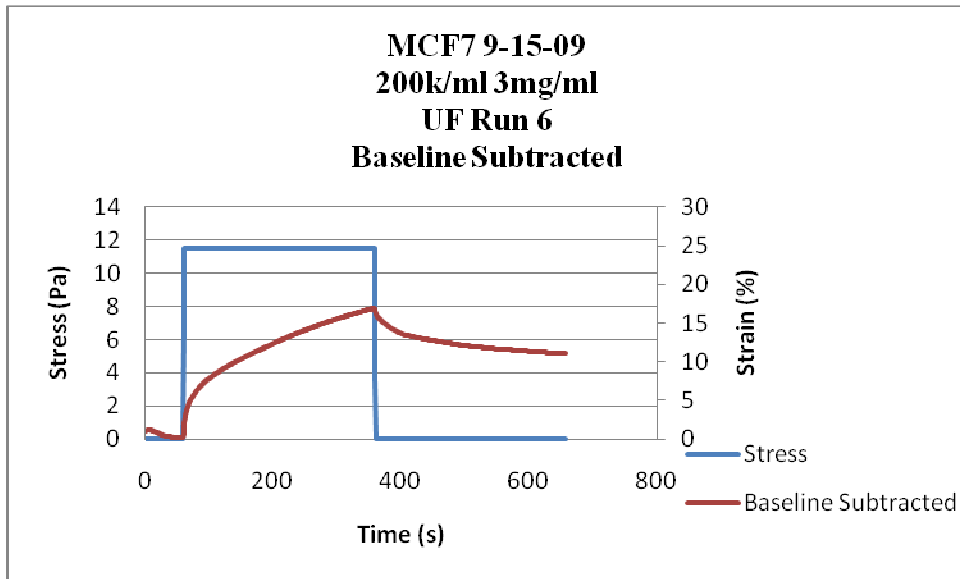
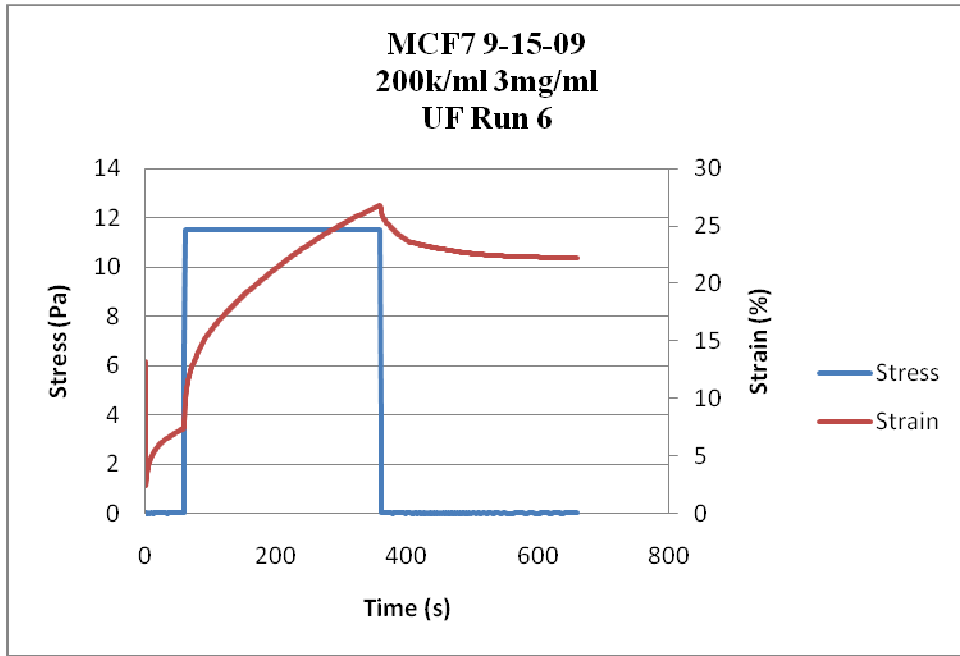
## APPENDIX F

### MCF-7 UNFROZEN NONLINEAR REGRESSION ANALYSIS SAMPLE CALCULATION

MCF-7 UF Sample Calculation					t=0 to 50	
		New time	Strain	Stress	Strain/Stress	Equation
		s	absolute	Pa		$e(t)/s_o = (1/E_o) * (1 - (E_1/(E_o + E_1)) * \exp(-t/(N_1 * (E_o + E_1)/(E_o * E_1)))) + (t/N_o)$
E_o	144.3687151	0	0	0	0	0.00E+00
E_1	8943.442251	1	0.00748	6.904	0.00108343	6.61E-04
η1	1685.835236	2	0.02147	11.51	0.001865334	1.17E-03
ηo	5.46E+05	3	0.02758	11.51	0.002396177	1.63E-03
ta	11.86578878	4	0.03222	11.51	0.002799305	2.06E-03
		5	0.03606	11.51	0.003132928	2.45E-03
		6	0.03911	11.51	0.003397915	2.82E-03
Mean_of_y	0.005347064	7	0.04167	11.51	0.00362033	3.15E-03
df	47	8	0.04393	11.51	0.003816681	3.45E-03
SE	0.000344328	9	0.04601	11.51	0.003997394	3.73E-03
R^2	0.960695048	10	0.04788	11.51	0.004159861	3.99E-03
critical t	2.01174048	11	0.04955	11.51	0.004304952	4.23E-03
CI	0.000692699	12	0.05113	11.51	0.004442224	4.45E-03
		13	0.05253	11.51	0.004563858	4.65E-03
		14	0.05393	11.51	0.004685491	4.83E-03
		15	0.05523	11.51	0.004798436	5.00E-03
		16	0.05654	11.51	0.00491225	5.16E-03
		17	0.05765	11.51	0.005008688	5.30E-03
		18	0.05887	11.51	0.005114683	5.43E-03
		19	0.05998	11.51	0.005211121	5.55E-03
		20	0.06119	11.51	0.005316247	5.66E-03
		21	0.06232	11.51	0.005414422	5.77E-03
		22	0.06345	11.51	0.005512598	5.86E-03
		23	0.06447	11.51	0.005601216	5.95E-03
		24	0.06551	11.51	0.005691573	6.02E-03
		25	0.06653	11.51	0.005780191	6.10E-03
		26	0.06747	11.51	0.005861859	6.16E-03
		27	0.06842	11.51	0.005944396	6.23E-03
		28	0.06936	11.51	0.006026064	6.28E-03
		29	0.0702	11.51	0.006099044	6.33E-03
		30	0.07105	11.51	0.006172893	6.38E-03

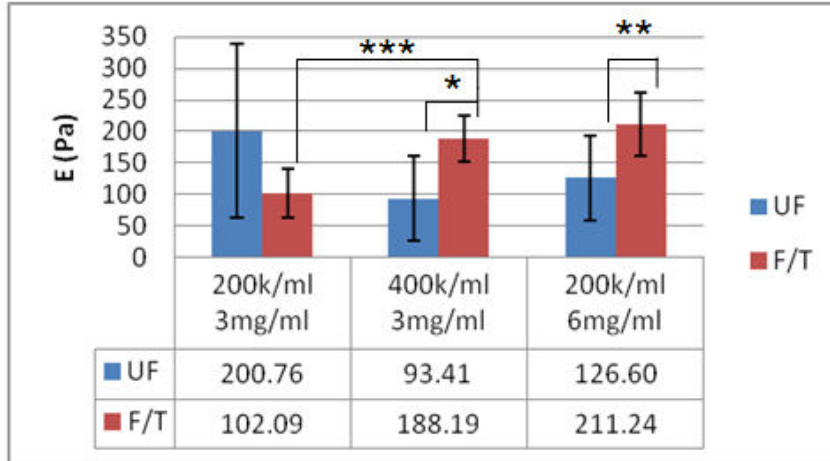
31	0.0719	11.51	0.006246742	6.43E-03
32	0.07275	11.51	0.006320591	6.47E-03
33	0.0735	11.51	0.006385752	6.50E-03
34	0.07426	11.51	0.006451781	6.54E-03
35	0.07509	11.51	0.006523892	6.57E-03
36	0.0757	11.51	0.00657689	6.60E-03
37	0.07619	11.51	0.006619461	6.62E-03
38	0.07687	11.51	0.00667854	6.65E-03
39	0.07745	11.51	0.006728931	6.67E-03
40	0.07803	11.51	0.006779322	6.69E-03
41	0.07861	11.51	0.006829713	6.71E-03
42	0.0793	11.51	0.006889661	6.73E-03
43	0.07987	11.51	0.006939183	6.74E-03
44	0.08045	11.51	0.006989574	6.76E-03
45	0.08104	11.51	0.007040834	6.77E-03
46	0.08163	11.51	0.007092094	6.78E-03
47	0.08222	11.51	0.007143354	6.80E-03
48	0.0828	11.51	0.007193745	6.81E-03
49	0.08338	11.51	0.007244136	6.82E-03
50	0.08396	11.51	0.007294526	6.83E-03



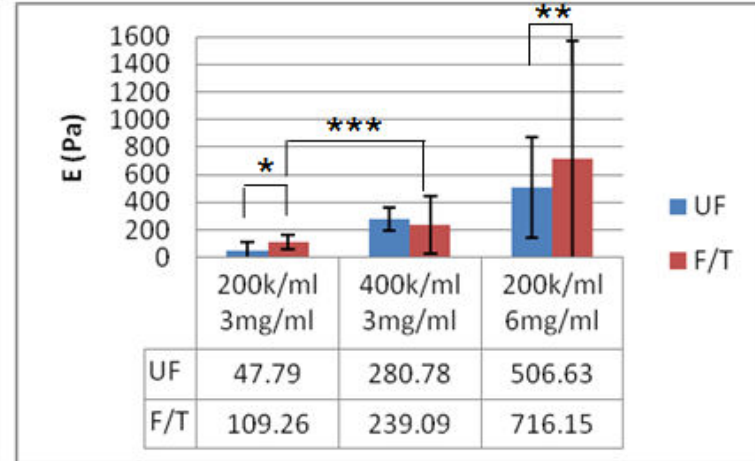


APPENDIX G

VISCOELASTIC DATA

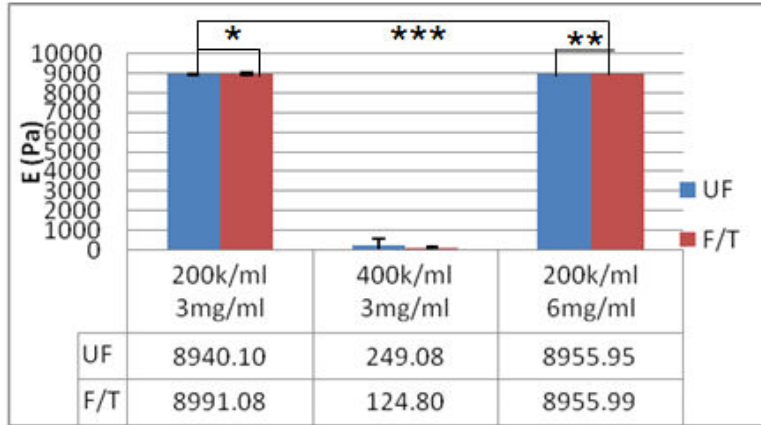


(a)

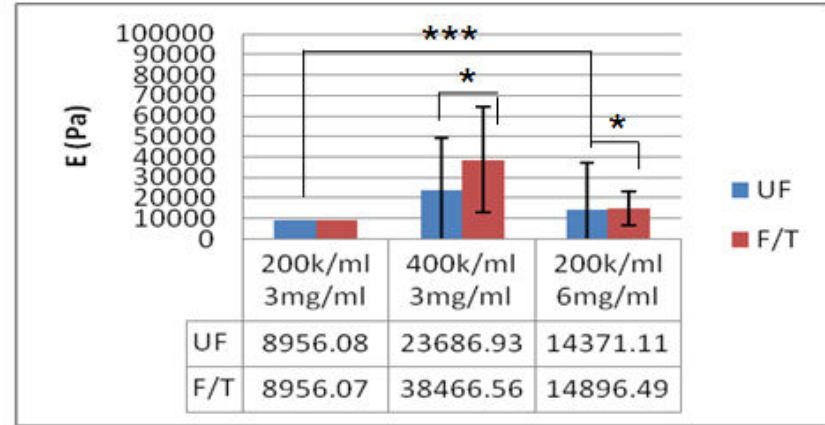


(b)

Plot of Elastic 0 Results a) MCF-7 (\*P=.09) (\*\*P=0.16) (\*\*P=0.04) b) BR5 (\*P=0.25) (\*\*P=0.72) (\*\*P=0.36).

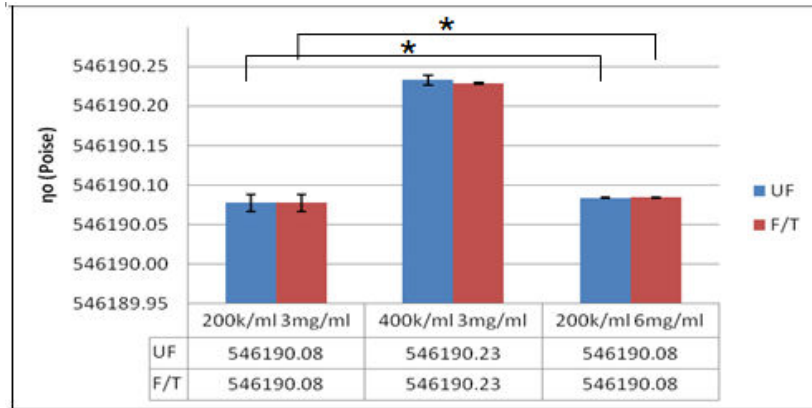


(a)

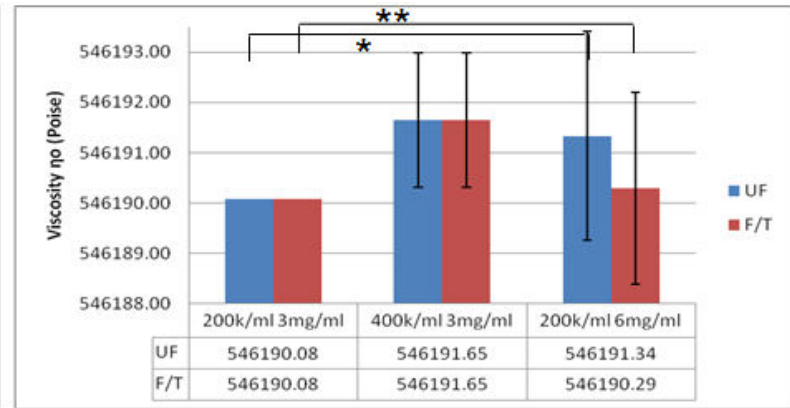


(b)

Plot of Elastic 1 Results a) MCF-7 (\*P=0.32) (\*\*P=0.78) (\*\*P=0.2) b) BR5 (\*P=0.52) (\*\*P=0.97) (\*\*P=0.70).



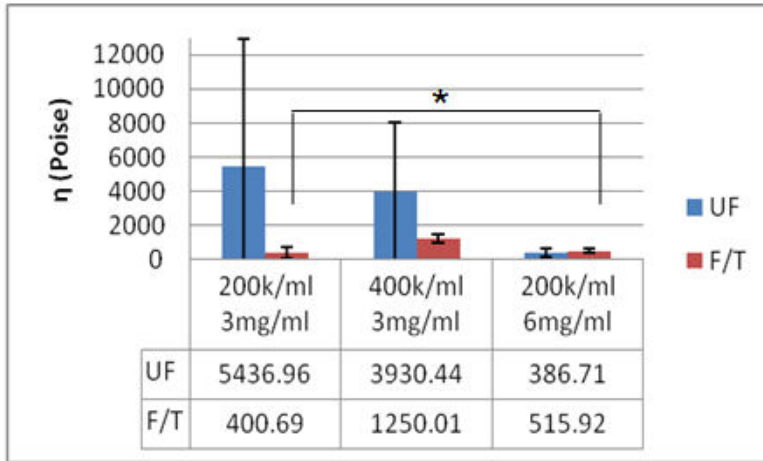
(a)



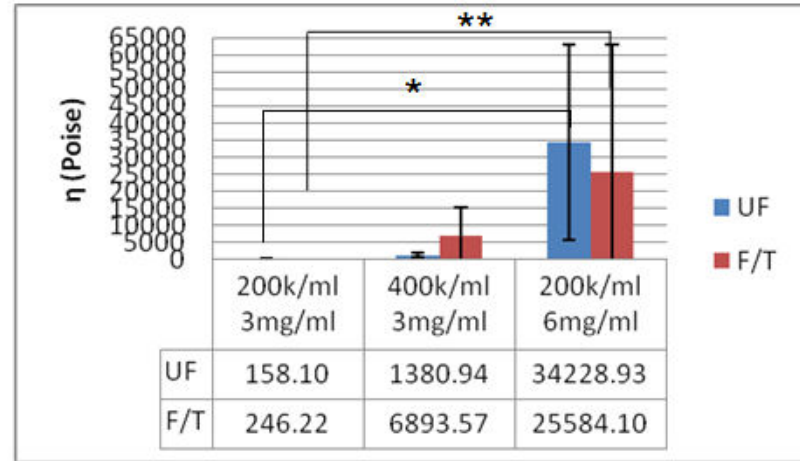
(b)

Plot of Viscosity 0 Results a) MCF-7 (\*P=0.35) b) BR5 (\*P=0.11) (\*\*P=0.86).



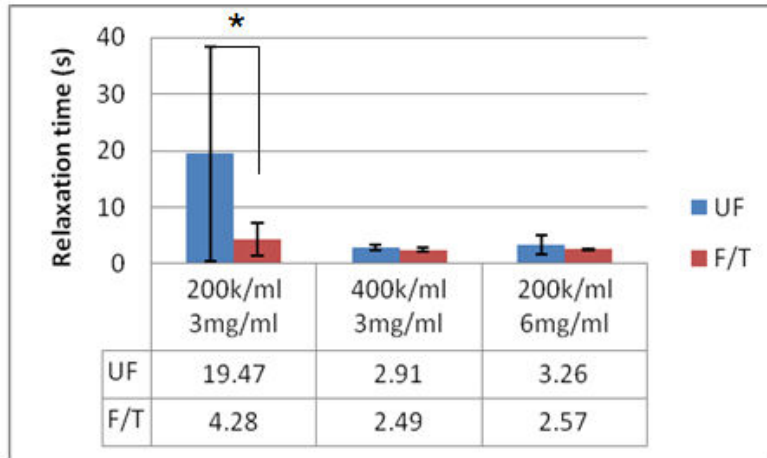


(a)

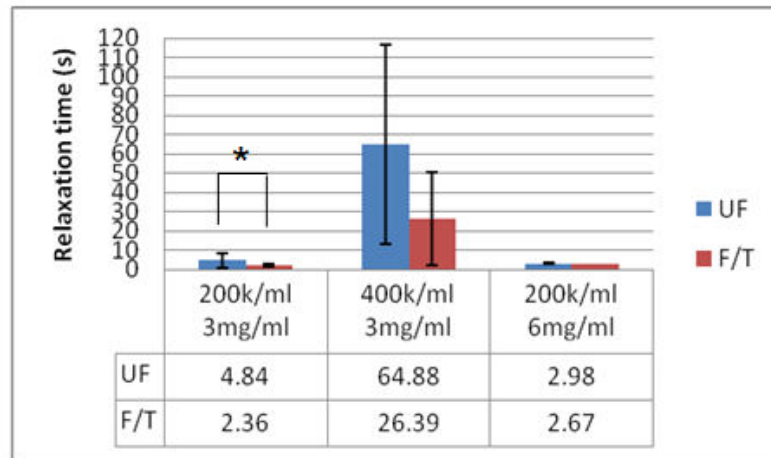


(b)

Plot of Viscosity 1 Results a) MCF-7 (\*P=0.59) b) BR5 (\*P=0.11) (\*\*P=0.30).



(a)



(b)

Plot of Relaxation Time Results a) MCF-7 (\*P=0.24) b) BR5 (\*P=0.33).

## REFERENCES

- [1] Devireddy, R. V., Smith, D. J., and Bischof, J. C., 2002, "Effect of Microscale Mass Transport and Phase Change on Numerical Prediction of Freezing in Biological Tissues," *Journal of Heat Transfer*, **124**(2) pp. 365-374.
- [2] Cui, Z. F., Dykhuizen, R. C., Nerem, R. M., 2002, "Modeling of Cryopreservation of Engineered Tissues with One-Dimensional Geometry," *Biotechnology Progress*, **18**(2) pp. 354-361.
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